Pathogenic Potentials of Clinical MRSA Isolates from the UK and Nigeria: A Comparative Study

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Authorship statement

Except where otherwise stated, this thesis is the result of my own research studies. I have also presented some of the findings presented in this thesis at various national and international conferences while some make up part of the results published in:

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Abstract

Methicillin-resistant *S. aureus* (MRSA) are a major problem in both clinical and community settings. They have the potential to produce invasive infections and are now most often multi-resistant. There is an indication that African *S. aureus* may be more virulent than those from other parts of the world. Thus, the primary aim of this study was to determine if Nigerian MRSA were more virulent than UK MRSA.

Polymerase chain reaction characterisation of all the MRSA isolates under study revealed variability in the distribution of virulence factors among the isolates. With the aid of two-dimensional electrophoresis, variation was observed in the intracellular protein profiles of MRSA from the same and different lineages. Antibiogram profiling of the isolates, using antibiotic disc susceptibility and minimum inhibitory concentration assays, showed that higher proportions of Nigerian MRSA were resistant to more antibiotics compared to UK MRSA. An assessment of the levels of interaction between the MRSA isolates with cell lines (KB and A549) showed that no significant differences existed between the means of the levels of interaction (association, invasion) of the UK and Nigerian MRSA with the cell lines. A further investigation of the cytotoxic effects of MRSA on cell lines showed that with just one exception, means of the toxic effects of live Nigerian MRSA, on both cell lines, were significantly greater than those of live UK MRSA on the cell lines, 6 and 24 hrs post-infection. Assessment of the anti-MRSA potential of five novel iron-chelators (iChs) revealed that the iCh with the highest molecular weight had the greatest inhibitory effect on MRSA.

Although findings from this work indicate that Nigerian MRSA are more virulent than UK MRSA, more work still needs to be done to conclusively determine if African *S. aureus* are indeed more virulent than those from other parts of the world.

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Abbreviations

2-DE: Two-dimensional electrophoresis

ABR: Antibiotic resistance

agr: Accessory Gene Regulator

AI: Autoinducer

AIDS: Acquired immune deficiency syndrome

AIP: Autoinducing peptide

BIR: Bacterial inhibition rate

bp: Base pair

BSI: Blood stream infection

CA-MRSA: Community-acquired methicillin-resistant *Staphylococcus aureus*

CC: Clonal complex

***ccr*:** Cassette chromosome recombinase

CFU: Colony forming unit

CHIPS: Chemotaxis Inhibitory Protein of *Staphylococcus aureus*

CLED: Cystine lactose electrolyte deficient

Clf: Clumping factor

CM: Conditioned medium

CP: Capsular polysaccharide

DMEM: Dulbecco's Modified Eagle's Medium

DMSO: Dimethyl sulfoxide

dNTP: deoxyribonucleotide triphosphate

ECM: Extracellular matrix

EDTA: Ethylenediaminetetraacetic acid

EMEM: Minimum Essential Medium Eagle

ent: Enterotoxin

ESKAPE: *Enterococcus faecium*, *Staphylococcus aureus, Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species

EUCAST: European Committee on Antibiotic Susceptibility Testing

FBS: Fetal Bovine Serum

FITC: Fluorescein Isothiocyanate

Fn: Fibronectin

FnBP: Fibronectin binding protein

HAI: Hospital acquired infection

HA-MRSA: Healthcare-associated methicillin-resistant *Staphylococcus aureus*

HIV: Human immunodeficiency virus

HK: Heat-killed

HPA: Health protection agency

hr: Hour

iCh: Iron-chelator

IE: Infective endocarditis

IEF: Isoelecric focusing

IPG: Immobilized pH gradient

kbp: Kilobase pair

LA-MRSA: Livestock-associated methicillin-resistant *Staphylococcus aureus*

MIC: Minimum inhibitory concentration

min: Minute

MLST: Multilocus sequence typing

MOI: Multiplicity of infection

mrMRSA: Multi-resistant MRSA

MRSA: Methicillin-resistant *Staphylococcus aureus*

MSCRAMMS: Microbial surface components recognizing adhesive matrix

MSSA: Methicillin-susceptible *Staphylococcus aureus*

NCTC: National Collection of Type Cultures

NEAA: Non-essential amino acids

NGS: Next generation sequencing

NHS: National Health Service

nmrMRSA: Non-multi resistant methicillin-resistant *Staphylococcus aureus*

NPPC: Non-professional phagocytic cells

OD: Optical density

ONS: Office for National Statistics

PBP: Penicillin-binding proteins

PCR: Polymerase chain reaction

PFGE: Pulsed field gel electrophoresis

PHE: Public health England

pI: Isoelectric point

pmol: Picomole

PS: Phosphatidylserine

PSM: Phenol soluble modulins

PVL: Panton Valentine Leukocidin

QS: Quorum sensing

Rot: Repressor of toxins

SAB: *Staphylococcus aureus* bacteraemia

***sae*:** *Staphylococcus aureus* accessory element

SaeRS: *Staphylococcus aureus* exoprotein expression

SAg: Superantigen

SAIE: *S. aureus* infective endocarditis

***sarA*:** Staphylococcal accessory regulator

SCC*mec*: Staphylococcal cassette chromosome *mec*

SE: Staphylococcal enterotoxins

secs: Seconds

***spa*:** *Staphylococcus aureus* protein A

SSI: Surgical site infection

SSTI: Skin and soft tissue infection

ST: Sequence type

TAE: Tris-acetate-EDTA

TMP-SMX: Trimethoprim/sulfamethoxazole

TSST-1: Toxic shock syndrome toxin 1

UK: United Kingdom

VNTR: Variable number tandem repeat

WGS: Whole genome sequencing

Chapter One

Literature Review

1.1. Introduction

Staphylococcus aureus is a major human pathogen (Tong *et al*., 2015; Rong *et al*., 2017) known to cause both community-acquired and nosocomial infections (Uhlemann *et al.*, 2014; Rong *et al*., 2017). It is a highly virulent and dangerous bacterium responsible for many human infections worldwide (Lowy, 1998). Some characteristics that help account for *S. aureus*'s success as a versatile human pathogen include its genetic diversity and ability to acquire new exogenous genes, its ability to establish asymptomatic carriage, a remarkable propensity to acquire resistance to multiple antimicrobial agents and its virulence and quorum sensing mechanisms (Moellering, 2011). *Staphylococcus aureus* infections range from moderately severe infections of the skin or respiratory tract to life-threatening diseases such as necrotising pneumonia, necrotising fasciitis, osteomyelitis, endocarditis, toxic shock syndrome (Loffler *et al*., 2005; Otto, 2012; Tattevin *et al*., 2012; Reddy *et al*., 2017) and infections associated with indwelling medical devices (Kong *et al*., 2016).

A major problem, physicians have to face when treating *S. aureus* infections is resistance to antibiotics (Otto, 2012). The appearance and global spread of methicillin-resistant *S. aureus* (MRSA) has become one of the most serious contemporary challenges to the treatment of hospital-acquired infections (Crisostomo *et al*., 2001; Al-Zoubi *et al*., 2015). MRSA have a uniquely effective drug-resistance mechanism that can protect them against members of the β-lactam family of antibiotics (Crisostomo *et al*., 2001; Otto, 2012; Dordel *et al*., 2014; Al-Zoubi *et al*., 2015); with the β-lactam antibiotics ceftobipirole and ceftaroline being notable exceptions (Monecke *et al.*, 2016). The emergence and dramatic increase of community acquired MRSA infections in otherwise healthy individuals with no risk factors (such as recent hospitalisation or surgery, dialysis, indwelling percutaneous medical devices and catheters) has also become an issue of increasing concern (Naimi *et al*., 2003; Boucher and Corey, 2008; Loffler *et al*., 2010). Therapeutic options available for MRSA infections are limited because MRSA has acquired

resistance to multiple antibiotic classes (Loffler *et al*., 2010; Chatterjee and Otto, 2013; Kaur and Chate, 2015). Therefore, it is important to better understand *S. aureus* (both methicillin-susceptible and – resistant) in order to comprehend and potentially predict trends in antibiotic-resistance patterns as well as to establish adequate infection control measures.

1.2. Concept of host-pathogen interactions

The lifestyle of a pathogenic bacteria revolves around locating a host, finding a colonisation niche, initiating and establishing an infection, and dispersal to a new host respectively (Gordon *et al*., 2013). Colonisation establishes the organism at the portal of entry (OECD, 2016) and has historically been defined as:

1. Appearance or increase in numbers of a particular invasive bacterial species in the resident microflora (Weinstein and Musher, 1969);
2. Presence of microorganisms which do not belong to the normal flora of the host but do not inflict local damage to the host (von Graevenitz, 1977);
3. Implantation of a microbe at a site, such as multiplication of staphylococci in the anterior nares (John Wiley and Sons, 1986);
4. Multiplication of an organism on a body surface without evoking an immune response (Evans, 1998);
5. An agent whose presence in a host does not cause a specific immune response or infection (Osterholm *et al*., 2000);

However, Casadevall and Pirofski, 2001, put forward that colonisation is characterised by microbial replication that may induce host damage and trigger a microbe-specific immune response which in turn could eradicate or contain the microbe. They explained that the state of colonisation is eliminated if the host immune response, antimicrobial therapy and or vaccination succeed in eradicating the microbe; if, however, elimination is not successful, a state of persistence

may ensue (Casadevall and Pirofski, 2001). Progressive damage resulting from this state may lead to disease and death (Casadevall and Pirofski, 2001).

While authors like Kolmer, 1924, and Thomson and Smith, 1994, consider an infection to be the outcome of a tissue invasion, authors like Sparling, 1983, Wilson and Miles, 1975, have defined infection to include initial contact between parasite and host. Infection was also defined by Henrici, 1934 as being the invasion of the body tissues by microorganisms resulting in disease, while Meyer *et al*., 1974, defined infection as a process in which an organism enters, establishes itself, and multiplies in the host (not in others). Furthermore, Evans, 1998, defined infection as the deposition, colonisation, and multiplication of a microorganism in a host; usually accompanied by a host response. However, in a bid to overcome what they saw as the inadequacy of available definitions of infection, Casadevall and Pirofski, in the year 2000 proposed that infection be defined as the acquisition of a microbe by host.

Though commonly thought of as a microbial property (Methot and Alizon, 2014), virulence can be defined as the relative capacity of a microbe to cause damage which is always measured relative to a standard, such as another microbe or host (Casadevall, 2017). Therefore, rather than being an independent variable, virulence is a dependent variable that is contingent on the availability of a susceptible host and the context/nature of the host-microbe interaction. (Casadevall and Pirofski, 2001; Casadevall, 2017). Thus, so-called virulent microbes are avirulent in hosts with specific immunity, and microbes that are usually avirulent cause disease in immunocompromised hosts (Casadevall and Pirofski, 2001; Primrose and Twyman, 2008; Aggarwal, 2010). An example is acquired immune deficiency syndrome (AIDS), in which the $CD4^+$ helper lymphocytes are progressively decimated by human immunodeficiency virus (HIV) (Peterson, 1996; Aggarwal, 2010). Thus, virulence is "a host-centred measure of a phenomenon that is neither host nor parasite but of the host parasite complex" (Poulin and Combes, 1999; Casadevall and Pirofski, 2001;

Knowles *et al*., 2018). In addition to the definition of virulence given above, other definitions of virulence have previously been put forward by several disciplines in the field of pathology i.e.

1. The disease producing power of an organism. Degree of pathogenicity within a group or species (Steinhaus and Martignoni, 1970);
2. Degree of pathogenicity against a specific species host in controlled conditions within a group or species of microorganisms (Aizawa, 1971);
3. The disease producing power of the pathogen, the ability to invade and injure the host's tissues (Tanada and Fuxa, 1987);
4. The number of dead individuals relative to the number infected (Thomas and Elkinton, 2004).

All these definitions, aside from the one by Thomas and Elkinton, 2004, indicate virulence as a measure, i.e. the degree, of pathogenicity; thus, for a given host and pathogen, virulence is variable e.g., due to strain or environmental effects (Shapiro-Ilan *et al*., 2005).

Although host–microbe interactions can generally be classified as symbiotic, commensal, or pathogenic, these divisions should be viewed as a homeostasis and this kind of equilibrium is constantly evolving (Niu *et al*., 2013). The damage response framework (DRF) of host-microbe interactions, developed by Casadevall and Pirofski, 1999, is based on the core principle that there are no exclusive pathogens, commensals, or opportunists, rather, microbial pathogenesis requires a microbe and a host to interact, with the relevant outcome being damage to the host (Jabra-Rizk *et al*., 2016). The ensuing damage results from microbial and/or host factors; while the host response contributes to microbe-mediated damage, host damage can stem from either weak or strong responses to microbes (Jabra-Rizk *et al*., 2016). Table 1.1 shows the classification of pathogens based on the damage response framework.

Table 1.1: Classification of pathogens based on the damage response framework (Jabra-Rizk *et al*., 2016)

Class	Description
1	pathogens that cause damage only in the setting of weak immune responses
2	pathogens that cause damage either in hosts with weak immune responses or in the setting of normal immune responses
3	pathogens that cause damage in the setting of appropriate immune responses and produce damage at both ends of the continuum of immune responses
4	pathogens that cause damage primarily at the extremes of both weak and strong immune responses
5	pathogens that cause damage across the spectrum of immune responses, but damage can be enhanced by strong immune
6	pathogens that can cause damage only as a result of strong immune responses

Pathogens utilise virulence factors in the process of host-pathogen interactions (Niu *et al*., 2013). These virulence factors protect the pathogen from host defences while facilitating the colonisation and subsequent destruction of host cells and tissues liberating nutrients which sustain pathogen growth (Gordon *et al*., 2013). Despite the presence of an extensive array of virulence factors, *S. aureus* can colonise without harming the host (Brown *et al*., 2014; Kavanaugh and Horswill, 2016). Nevertheless, many of these virulence factors are being produced at some level based on the presence of antibody responses (Kavanaugh and Horswill, 2016).

In some instances, host immune response to infection is responsible for much of the tissue damage rather than bacterial factors (Peterson, 1996; Machado *et al*., 2004; Pechous, 2017). Toxic agents, e.g. proteinases, cationic polypeptides, cytokines, and reactive oxygen species, released from the lymphocytes, macrophages, and polymorphonuclear neutrophils infiltrating the site of infection are responsible for tissue damage in these infections (Peterson, 1996; Pechous, 2017). The host response is often so intense that host tissues are destroyed, and a classic example of this host response-mediated pathogenesis is seen in leprosy, (Peterson, 1996; Fonseca *et al*., 2017).

1.3. *Staphylococcus aureus* colonisation and infection

Staphylococcus aureus is a frequent asymptomatic coloniser of humans (Chatterjee and Otto, 2013). Colonisation provides a reservoir from which bacteria can be introduced when host defences are breached (Gordon and Lowy, 2008; Kong *et al*., 2016) which could be by shaving, aspiration, surgery or insertion of indwelling catheters (Gordon and Lowy, 2008). Therefore, *S. aureus* colonisation is a risk factor for subsequent infection caused by the colonising clone (von Eiff *et al*., 2001; Chatterjee and Otto, 2013; Schaumburg *et al*., 2014; Kong *et al*., 2016). Colonisation also allows *S. aureus* to be transmitted among individuals in both healthcare and community settings (Gordon and Lowy 2008). Other risk factors for acquisition of MRSA infection in the community include; direct skin-to-skin contact with an infected person, contact with contaminated fomites in household or public settings (Fridkin *et al*., 2005; Miller and Diep, 2008) and poor personal hygiene (Chatterjee and Otto, 2013).

The anterior nares are the main ecological niche for *S. aureus* carriage in humans (Gordon and Lowy, 2008; Otto, 2012; Kong *et al*., 2016). Humans were previously assigned to either of three groups with regards *S. aureus* nasal carriage, i.e. persistent carriers (~20% of individuals in the population), intermittent carriers (~30% of individuals in the population) and the non-carriers (~50% of individuals in the population) (Wertheim *et al*., 2005; Gordon and Lowy, 2008). However, the study by van Belkum and his colleagues in 2009 revealed that host-*S. aureus* interactions are highly specific and that so called intermittent and non-*S. aureus* carriers have similar elimination kinetics as well as similar anti-staphylococcal antibody patterns (Belkum *et al*., 2009). As such, only two categories of nasal carriage exist in humans i.e. the persistent carriers and others (van Belkum *et al.*, 2009). While the most common site of MRSA colonisation is the anterior nares, *S. aureus* (including MRSA) may also be present in the throat, axilla, rectum, groin, or perineum, and frequently colonises more than one site (Mermel *et al*., 2011; Albrechtet *et al*.,

2015). Recent studies by Albrecht *et al*., 2015 and Kumar *et al*., 2015 suggest that colonisation of the throat is more prevalent than colonisation of the nose, and that checking only the nose would fail to detect a significant portion of colonised persons.

An association between disease and *S. aureus* nasal carriage had been noted as far back as 1931 (Wertheim *et al*., 2005) and several studies have confirmed that most *S. aureus* infections originate from strains that colonise the nose (von Eiff *et al*., 2001; Wertheim *et al*., 2005; van Belkum, 2016). Nasal carriage has been shown to differ among ethnic groups (Adesida *et al*., 2007; Otto, 2012; Sollid *et al*., 2014), age groups (Lamikanra *et al*., 1985; Kluytmans *et al*., 1997; Adesida *et al*., 2007; Sollid *et al*., 2014) and gender (Lamikanra *et al*., 1985; Sollid *et al*., 2014). *Staphylococcus aureus* carriage rates are higher in patients with certain diseases (Lowy, 1998; Otto, 2012) such as patients with acquired immune deficiency syndrome (AIDS), surgical patients and patients with type-1 diabetes (Lowy, 1998; Otto, 2012; Alexander *et al*., 2017). These emphasise the importance of host factors in determining *S. aureus* colonisation (Otto, 2012). Though complex and incompletely understood, the basis for *S. aureus* colonisation appears to involve the host's contact with *S. aureus*, the ability of *S. aureus* to adhere to host cells and *S. aureus*'s ability to evade the immune response (Gordon and Lowy, 2008; Otto, 2008; Otto, 2012).

In the hospital, the ultimate source of MRSA infection are the patients or hospital personnel that carry MRSA, but, contaminated fomites and medical devices may also play a role as intermediate sources of MRSA infection (Otto, 2012). Underlying conditions such as compromised immune system (Chatterjee and Otto, 2013) as well as invasive approaches for the diagnostic and therapeutic management of infections (Amaral *et al*., 2005; Planet *et al*., 2013) are risk factors for MRSA infections during hospitalisation. As with other multi drug resistant bacteria (Hoang *et al*., 2018), antibiotic use at both the patient and institutional level is also a risk for MRSA colonisation and subsequent infection (Shorr, 2007; Shet *et al*., 2009).

1.4. Virulence factors expressed by *S. aureus*

A key factor affecting the severity and outcome of any infection is the virulence potential of the infecting organism (Laabei *et al*., 2014). *Staphylococcus aureus* has an extensive pool of virulence factors, with both freely secreted and surface-bound factors playing a role in the pathogenesis of infection (Foster, 2005; Gordon and Lowy, 2008). The virulence factors expressed by *S. aureus* can be broadly grouped into being either adhesins, factors that facilitate adherence to and invasion of host tissues (Collins *et al*., 2010; Foster *et al*., 2014), or toxins, factors that cause specific tissue damage in the host (Lowy, 1998; Gordon and Lowy, 2008; Otto, 2010). Another group of genes expressed by *S. aureus* which also facilitate infection are the immune-modulators. They are proteins that interfere with host immunity preventing defence against and clearance of infection (Collins *et al*., 2010). It is worth noting that among staphylococci, a virulence factor may have several functions in pathogenesis and multiple virulence factors may perform the same function (Gordon and Lowy, 2008). The distribution of some virulence factors in *S. aureus* is related to clonal type, whereas the presence of others is unrelated to genetic background (Peacock *et al*., 2002).

Some key virulence factors responsible for the pathogenic success of *S. aureus* are discussed below.

1.4.1. Surface proteins

Surface proteins play multiple roles in *S. aureus* pathogenesis (Otto, 2012). They function in cell wall metabolism, serve to bind to host tissue, facilitate internalisation and immune evasion and are involved in bacterial aggregation and biofilm formation (Foster and Hook, 1998; Foster *et al*., 2014). Based on the presence of motifs that have been defined by structure-function analysis, surface proteins are classified into four groups: (a) The microbial surface components recognising adhesive matrix molecules (MSCRAMMs); (b) The tandemly repeated three-helical bundles; (c)

The near iron transporter (NEAT) motif family; and (d) The G5–E repeat family (Foster *et al*., 2014; Jan-Roblero *et al*., 2016). The most prevalent group of the *S. aureus* surface proteins are the MSCRAMMs (Foster *et al*., 2014) which mediate adherence to host tissues (Gordon and Lowy, 2008) by interacting with extracellular matrix components (Alexander *et al*., 2003). They are actively synthesised during the exponential growth phase, coinciding with the tissue-binding and colonisation phases of infection (Cheung *et al*., 2004). Specific MSCRAMMs are responsible for localisation of *S. aureus* to particular tissues, and include collagen-binding adhesion (Patti *et al*., 1992), elastin binding adhesion (Park *et al*., 1996), fibrinogen-binding proteins (Cheung *et al*., 1995), fibronectin-binding proteins (FnBPs) (Hynes, 1990; Jonsson *et al*., 1991), and a broad-specificity adhesin (MAP) that facilitates low-affinity binding of *S. aureus* to several proteins, including collagen, vitronectin, bone sialoprotein, fibronectin, fibrinogen, and osteopontin (McGavin *et al*., 1993). *Staphylococcus aureus* interaction with host cells via MSCRAMMs can induce cytoskeletal rearrangement, signal transduction and tyrosine kinase activity (Dziewanowska, 1999). MSCRAMMs also appear to play a key role in initiation of endovascular infections, bone and joint infections, and prosthetic-device infections (Gordon and Lowy, 2008). Once *S. aureus* adheres to host tissues or prosthetic materials, it is able to grow and persist in various ways. Notable examples of MSCRAMMS are the clumping factors (*Clfs*) A and B. *Clf*A and *Clf*B are fibrinogen-binding proteins primarily responsible for the clumping of *S. aureus* in fibrinogen solutions and *S. aureus* adherence to fibrinogen substrata (McDevitt *et al*., 1994; Paharik and Horswill, 2016). It is important to note that *ClfA* unlike other MSCRAMMS is produced during the stationary phase of growth (O'Brien *et al*., 2002; Bischoff *et al*., 2004).

Staphylococcus aureus protein A (*spa*) is the only *S. aureus* surface protein in the tandemly repeated three-helical bundles group (Foster *et al.*, 2014; Paharik and Horswill, 2016). *Spa* blocks fragment crystallisable (Fc)-receptor mediated phagocytosis of *S. aureus* (Rooijakkers and Van Strijp, 2007) by binding the Fc portion of immunoglobulin G (IgG) (Deisenhofer, 1981) and

leaving the Fab (fragment antigen binding) portions free to combine with specific antigens (Parija, 2009). In addition to being anti-opsonic, *spa*'s ability to cross-link V_H3 type B cell receptors (surface IgM), resulting in proliferative supraclonal expansion as well as apoptotic collapse of the activated B cells makes it a potent immunomodulatory molecule (Goodyear and Silverman, 2003; Foster, 2005).

Near iron transporter (NEAT) motif proteins (the iron-regulated surface determinant (Isd) proteins) are able to capture haem from haemoglobin, thereby helping bacteria survive in the host, where iron is limited (Foster *et al*., 2014; Jan-Roblero *et al*., 2017). Isd transports haem to a membrane transporter which then transfers the haem into the cytoplasm, where it is degraded by haemoxygenases to release free iron (Villareal *et al*., 2008; Foster *et al*., 2014; Jan-Roblero *et al*., 2017). Furthermore, Isd protects *S. aureus* from the bactericidal fatty acids present in the sebum (Satoskar and Nadasdy, 2017). The *S. aureus* surface protein G (SasG) is a G5-E repeat protein known to promote biofilm formation (Foster *et al*., 2014; Jan-Roblero *et al*., 2017).

1.4.2. Biofilm formation

Biofilms are a structured community of bacteria enclosed in a self-produced polymeric matrix and adherent to an inert or living surface (Costerton *et al*., 1999; Miquel *et al*., 2016). They constitute a protected mode of growth that allows survival in a hostile environment (Costerton *et al*., 1999). In addition to the biofilm matrix impeding access of immune defences (e.g. macrophages) during infection, biofilm cells display increased tolerance to antibiotics i.e. a transient state in which normally susceptible bacteria enter an altered physiology that decreases sensitivity (Lister and Horswill, 2014). Furthermore, after the establishment of a biofilm, individual cells can disperse from the original biofilm and either seed new sites of infection or mediate an acute infection (Costerton *et al*., 1999; Lister and Horswill, 2014). Thus, biofilms may contribute to the persistence in infection and colonisation, recalcitrance of staphylococcal infections to treatment,

as well as spread of staphylococci in hospital and community settings (Yarwood *et al*., 2004; Otto, 2012).

The physiological status during *S. aureus* colonisation in the nose may be comparable in many aspects to that in biofilms. For instance, colonising or biofilm-forming *S. aureus* remain on or in human epithelia in relative silence as opposed to an aggressive status during acute *S. aureus* disease (Otto, 2012). However, whether *S. aureus* colonies in the nose can be considered biofilms is debatable (Otto, 2012).

1.4.3. Toxins

Toxins are proteins secreted by *S. aureus* into the extracellular matrix during the post-exponential and early stationary phases (Kong *et al*., 2016A). Their ability to lyse human cells causes local tissue damage facilitating immune evasion, release of nutrients, dissemination within a host, and transmission to others (Lowy, 1998; Gordon and Lowy, 2008; Otto, 2010). It is common to observe differences in the toxin repertoire between *S. aureus* clones because many *S. aureus* toxins are encoded on mobile genetic elements (MGEs) whose presence vary considerably between strains (Otto, 2012). Core-genome encoded toxins such as α-toxin, γ-toxin, PSMs and some leukocidins are produced by most strains (Otto, 2012) and differences in the expression of these core-genome encoded toxins may also give rise to differences in the pathogenic potential between *S. aureus* strains (Otto, 2012).

1.4.3.1. Superantigens (Pyrogenic Toxin Superantigens): Superantigens (SAgs) are a highly important family of exotoxins secreted by *S. aureus*. They overstimulate many immune processes that allow *S. aureus* to cause serious human illnesses (Spaulding *et al*., 2013). There exist at least 26 serologically distinct *S. aureus* SAgs (Tuffs *et al*., 2017) and all pathogenic strains of *S. aureus* secrete SAgs (Stach *et al*., 2014). Superantigens produced by *S. aureus* include toxic shock syndrome toxin-1 (TSST-1), staphylococcal enterotoxins (SEs) and SE-like (SE-*l*) SAgs

(Dinges *et al*., 2000; McCormick *et al*., 2001; McCormick *et al*., 2006; Lin and Peterson, 2010). Although the staphylococcal exfoliative toxins (causative agent of the staphylococcal scalded skin syndrome) are SAgs, they are milder compared to other SAgs like TSST-1 (Kong *et al*., 2016A). Genes that code for SAgs are carried on mobile genetic elements (MGEs such as bacteriophages, pathogenicity islands and plasmids) (Dinges *et al.*, 2000; Otto, 2012; Corredor *et al*., 2016) and like most proteins, they are produced primarily in the post-exponential phase of growth (Dinges *et al*., 2000). Superantigens exhibit properties such as superantigenicity, pyrogenicity and the ability to enhance susceptibility to Gram-negative lipopolysaccharide (LPS) up to 10^6-fold (Spaulding *et al*., 2013; Stach *et al*., 2014). Superantigenicity is the best characterised property of SAgs and it refers to their ability to stimulate proliferation of T lymphocytes without regard for the antigen specificity of these cells (Fleischer and Schrezenmeier, 1988; Marrack and Kappler, 1990; Bien *et al*., 2011). Superantigens are highly resistant to heat denaturation, desiccation, proteolysis and acid denaturation (McCormick *et al*., 2001; McCormick *et al*., 2006; Stach *et al*., 2014). This environmental stability and their biological toxicity has led to some SAgs (e.g. Staphylococcal enterotoxin B) being categorised as select agents of bioterrorism (Spaulding *et al*., 2013).

1.4.3.2. Cytotoxins: They are pore-forming toxins that form β-barrel pores in the membranes of target cells causing leakage (osmotic swelling) and inflammation of the cells and ultimately cell lysis (Foster, 2005; Lin and Peterson, 2010). Cytotoxins produced by *S. aureus* include the alpha (α), beta (β) and gamma (γ) toxins, the Phenol-soluble modulins (PSMs) and the Leukocidins (Panton-Valentine leukocidine (PVL), LukM, LukD/E and leukocidin) (Parija, 2009; Lin and Peterson, 2010; Stach *et al*., 2014). These toxins are structurally diverse and have various target specificity (i.e., erythrocytes, leukocytes and epithelial cells) (Lin and Peterson, 2010). By killing off neutrophils that are attempting to engulf and kill bacteria, cytotoxins contribute to the development of abscesses (Foster, 2005) which is believed to facilitate transmission between hosts (Collins *et al*., 2010). Some cytotoxins, i.e. PVL, γ-toxin, leukocidin E/D and leukocidin M/F-PV,

are bicomponent toxins as they comprise two separately secreted subunits (Foster, 2005; Lin and Peterson, 2010; Otto, 2014). Each individual subunit (fast-eluting (F) and slow-eluting (S) subunits) on its own lacks biological activity but they assemble into hexameric or heptameric oligomers with a strong affinity for leukocytes (Foster, 2005; Lin and Peterson, 2010). Panton-Valentine leukocidine (PVL) is toxic only for leukocytes while γ-toxin lyses both leukocytes and erythrocytes (Menestrina *et al*., 2003; Kaneko and Kamio, 2004). Alpha-toxin is active against various cells, including erythrocytes, leukocytes, epithelial cells, fibroblasts and platelets (Menestrina *et al*., 2003) but not neutrophils (Kong *et al*., 2016). Beta-toxin (sphingomyelinase C) hydrolyses sphingomyelin and as such the sensitivity of human cells to the toxicity of β-toxin depends upon the distribution of sphingomyelin on the membrane (Lin and Peterson, 2010). Although it lyses monocytes, β-toxin only lyses erythrocytes at low temperatures and is not cytolytic to granulocytes and lymphocytes (Kong *et al*., 2016A). The δ-toxin has been classified as a PSM that does not need a receptor for its haemolytic activity (Kong *et al*., 2016). Like other PSMs (PSMα1-4, PSM*mec*, PSMβ1-2), δ-toxin is haemolytic to bacterial protoplasts, erythrocytes, spheroplasts and various organelles (Kong *et al*., 2016A).

1.4.4. Capsular polysaccharide (CP)

Like many other pathogens, *S. aureus* expresses CP as a virulence factor to avoid opsonophagocytic killing during infection (O'Riordan and Lee, 2004; Nanra *et al*., 2013; Chan *et al*., 2014). Up to 13 serotypes of CPs have been identified in *S. aureus* (Chan *et al*., 2014) and capsulated strains are known to be more virulent than their non-capsulated counterparts (Parija, 2009). There are two major CP types, i.e. CP5 and CP8; all clinical *S. aureus* strains have the biosynthetic pathways for making either CP5 or CP8 (Nanra *et al*., 2013). Capsular polysaccharides have also been shown to enhance bacterial colonisation and persistence on mucosal surfaces (O'Riordan and Lee, 2004; Parija, 2009). Furthermore, the zwitterionic charge

of a CP (both positively and negatively charged) can induce abscess formation (Tzianabos *et al*., 2001; O'Riordan and Lee, 2004).

1.4.5. Chemotaxis Inhibitory Protein of *S. aureus* (CHIPS)

An immediate host response toward bacterial infection is the migration of leukocytes from circulation to the site of infection (De Haas *et al*., 2004). Detection of invading bacteria by neutrophils, triggered by excretion of formylated peptides and generation of C5a, is considered one of the earliest innate recognition events (Postma *et al*., 2004). Chemotaxis inhibitory protein of *S. aureus* is a potent inhibitor of immune cell recruitment and activation associated with inflammation, through binding and blocking the C5a receptor and the formylated peptide receptor (De Haas *et al*., 2004; Postma *et al*., 2004; Guerra *et al*., 2017). About 60% of *S. aureus* strains secrete CHIPS (Foster, 2005).

1.4.6. Enzymes and other virulence components

During infection, *S. aureus* produces numerous enzymes such as elastases, lipases, proteases and hyaluronidase, which enable it to invade and destroy host tissues and also facilitate the spread of infection to adjoining tissues (Lowy, 1998; Gordon and Lowy, 2008). The enzyme hyaluronidase facilitates the spread of *S. aureus* into tissues by hydrolysing the acidic mucopolysaccharides present in the matrix of connective tissues (Parija, 2009); the free form of the enzyme coagulase, a prothrombin activator, converts fibrinogen to fibrin (Lowy, 1998; Parija, 2009).

1.5. Regulation of virulence

In order to establish and maintain infection, *S. aureus* utilises an arsenal of virulence factors, which vary depending on its growth phase (Gomes-Fernandes *et al*., 2017). During the lag and early exponential phases, it produces cell wall associated factors that facilitate tissue attachment and evasion of the host immune system, allowing the bacteria to accumulate and form a biofilm (Wang and Muir, 2016). Once in the late exponential phase, the bacterial population begins to secrete a

spectrum of exoproteins, including proteases, haemolysins, and super-antigens, and at the same time down-regulates the cell wall-associated factors, leading to dispersion of the biofilm and the spread of the infection (Wang and Muir, 2016). This population density-dependent behaviour essentially describes two stages of the *S. aureus* life cycle, i.e., an adhesion phase and an invasion phase (Wang and Muir, 2016). Involvement of a diverse array of cell wall and extracellular components in the virulence of *S. aureus* implies that the pathogenicity of this bacterium is a complex process (Bien *et al*., 2011; Costa *et al*., 2013) that requires the coordinated expression of virulence factors during different phases of infection (Norvick and Jiang, 2003; Torres *et al*., 2010; Costa *et al*., 2013).

Staphylococcus aureus regulatory systems involved in the coordination of virulence respond to bacterial cell density and environmental cues (e.g. nutrient availability, temperature, pH, osmolarity, and oxygen tension) (Costa *et al*., 2013; Gordon *et al*., 2013). Quorum-sensing, one of the regulatory mechanisms that ensure timely adaptation of staphylococcal physiology to the environment (Le and Otto, 2015) ensures/allows efficient transition between the two stages of the *S. aureus* lifecycle (Wang and Muir, 2016). Quorum sensing is an intercellular communication process in which the bacterium produces a diffusive molecule, termed the autoinducer (AI), as an indicator of the local population density. (Wang and Muir, 2016). Autoinducers are detected by receptors that exist in the cytoplasm or in the membrane (Rutherford and Bassler, 2012). At low cell density, AIs diffuse away, and, therefore, are present at concentrations below the threshold required for detection (Rutherford and Bassler, 2012). However, when the cell density of *S. aureus* reaches a critical threshold the concentration of this signal accumulates and activates a QS regulatory cascade (Kavanaugh and Horswill, 2016).

Autoinducers accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and collectively alter gene

expression (Rutherford and Bassler, 2012). Quorum sensing controls genes that direct activities that are beneficial when performed by groups of bacteria acting in synchrony; virulence factor secretion, antibiotic production, sporulation and biofilm production are some processes controlled by QS (Rutherford and Bassler, 2012). In addition to activating expression of genes necessary for cooperative behaviours, detection of AIs results in activation of AI production (Rutherford and Bassler, 2012).

The accessory gene regulator (*agr*) is considered the prototype QS regulator system in Gram-positive bacteria (Le and Otto, 2015). The *agr* system is central in the control and regulation of virulence gene expression (Gomes-Fernandes *et al*., 2017). This autocatalytic operon is controlled in a cell density-dependent fashion through the production and sensing of the autoinducer, a small peptide named the AIP (autoinducing peptide) (Wang and Muir, 2016; Gomes-Fernandes *et al*., 2017). Accumulation of AIP triggers a series of signal transduction events that in turn activate expression of the *agr* locus (Balasubramanian *et al*., 2017).

The *agr* operon consists of divergent transcripts, RNAII and RNAIII, whose expression is driven by promoters designated P2 and P3, respectively (Montgomery *et al*., 2010; Gomes-Fernandes *et al*., 2017). The RNAII locus contains four genes i.e. *agrB*, *agrD*, *agrC* and *agrA* (Montgomery *et al*., 2010; Le and Otto, 2015). *AgrD* and *agrB* encode a quorum sensing system that results in the production of AIP that positively regulates *agr* expression (Montgomery *et al*., 2010). The *agrC* and *agrA* genes encode a two-component signal transduction system involving the *agrC* (histidine kinase) transmembrane protein and its associated response regulator, *agrA* (Yarwood and Schlievert, 2003; Montgomery *et al*., 2010; Le and Otto, 2015). When bacterial cell density surpasses a certain threshold (quorum), accumulated AIP binds to *agrC*, which then phosphorylates the response regulator *agrA* (Balasubramanian *et al*., 2017). Activated *agrA* binds to its own P2 promoter as well as the P3 promoter to drive the transcription of RNAIII (Le and

Otto, 2015; Balasubramanian *et al*., 2017). Furthermore, activated *agrA* binds the promoters controlling expression of the PSMα and PSMβ peptides (Le and Otto, 2015). While the *agrD* gene encodes the AIP precursor, the *agrD* gene encodes a transmembrane endopeptidase involved in the processing and export of the mature AIP (Le and Otto, 2015; Balasubramanian *et al*., 2017).

RNAIII is the intracellular effector molecule of the *agr* system responsible for the control of *agr* targets (Le and Otto, 2015). In addition to encoding the delta-haemolysin, RNAIII acts by binding to the *5*′ region of virulence-factor mRNAs and post-transcriptionally represses or activates virulence factors by either acting directly or by influencing their upstream regulators (Wang and Muir, 2016; Balasubramanian *et al*., 2017). RNAIII increases the expression of several secreted virulence factors including TSS toxin1 and other haemolysins (Yarwood and Schlievert, 2003; Montgomery *et al*., 2010). Other secreted toxins, such as enterotoxins B, C, and D, are only partially upregulated by RNAIII and can be made in high concentrations independently of *agr* (Yarwood and Schlievert, 2003). On the other hand, RNAIII decreases expression of several cell surface virulence factors involved in adhesion, including protein A and the fibronectin binding proteins (Yarwood and Schlievert, 2003; Montgomery *et al*., 2010).

Four different classes of AIP structures are known to exist in *S. aureus* i.e. types I (8 amino acids long), II (9 amino acids long), III (7 amino acids long) and IV (8 amino acids long); as they differ in only one amino acid, types I and IV are the most conserved and can function interchangeably (Gray *et al*., 2013; Jenul and Horswill, 2018). Ji *et al*., 1997, showed that AIPs can cross-inhibit the function of an *agrC* receptor from another *S. aureus* strain, in a mechanism termed '*agr* interference'. On the basis of this cross-inhibition, three functional AIP groups were defined, each of which can effectively cross-inhibit other *agr* systems within *S. aureus* in a mechanism of intraspecies signalling (Jenul and Horswill, 2018). In other words, the cognate AIP signal from a producing strain will cross-inhibit the *agrC* receptor (and in turn inhibit *agr* function) on a *S.*

aureus strain representing a different *agr* group (Jenul and Horswill, 2018). *Agr* cross-inhibition may drive evolutionary diversification among the staphylococci and has important implications for host infections (Geisinger *et al*., 2009).

The repressor of toxins (*Rot*), a virulence regulator that modulates activity of target promoters, is an RNAIII target (Balasubramanian *et al*., 2017). Due to the presence of few bacteria and low levels of AIP, the *agr* locus is thought to be inactive during the onset of infection and consequently, the level of *Rot* is high (Balasubramanian *et al*., 2017). *Rot* in turn upregulates the expression of immune evasion proteins and adhesins that help dodge first-line, innate immune defences (Balasubramanian *et al*., 2017). Increase in RNAII levels and the consequent inhibition of *Rot* translation, when infection is established and quorum is reached, leads to expression of toxins and exo-enzymes responsible for lysis of immune cells and tissue destruction (Balasubramanian *et al*., 2017). In addition to the AIP, *agr* can also be activated by other regulators, such as *sarA* (staphylococcal accessory regulator) or *SrrAB* (staphylococcal respiratory response AB) (Le and Otto, 2015), and environmental factors such as pH and nutrient availability (Le and Otto, 2015; Kavanaugh and Horswill, 2016).

In *S. aureus*, the upregulation of virulence factors by *agr* was found to contribute to pathogenesis in several infection models, including SSTIs, infective endocarditis, pneumonia, septic arthritis and osteomyelitis (Le and Otto, 2015). Down-regulation of PSMs and microbial surface components by *agr* has been implicated in enhanced biofilm formation and bacterial colonisation of indwelling medical devices (Le and Otto, 2015). Moreover, *agr* dysfunction has been associated with persistent *S. aureus* bacteraemia (Le and Otto, 2015; Gomes-Fernandes *et al*., 2017), decreased susceptibility to vancomycin and thrombin induced platelet microbicidal protein (Gomes-Fernandes *et al*., 2017). *Agr* dysfunctional strains generally have a higher biofilm capacity

and are more fit *in vitro* due to the large metabolic burden of having an active *agr* system (Gomes-Fernandes *et al*., 2017).

The staphylococcal accessory regulator A (*sarA*) and its homologues (e.g. *sarR, sarT, sarU* and s*arH1*) are an additional set of global regulators of *S. aureus* virulence (Yarwood and Schlivert, 2003; Costa *et al*., 2013; Jenul and Horswill, 2018). Moreover, *sarA* is the prototypical member of the *sarA* protein family in *S. aureus* (Jenul and Horswill, 2018). *SarA* is a 124-residue DNA binding protein encoded by the *sarA* locus, which consists of three overlapping transcripts, driven by three distinct promoters, P1, P3 and P2 (Cheung *et al*., 2008). *SarA* transcribed from these three promoters within the same locus, has been reported to be required for full *agr* transcription (Yarwood and Schlievert, 2003; Balasubramanian *et al*., 2017). *SarA* also affects virulence independently of *agr* by binding directly to promoters of genes encoding for many virulence factors (Balasubramanian *et al*., 2017). Phenotypically, the *sarA* locus promotes synthesis of fibronectin and fibrinogen binding proteins (for adhesion) and also toxins (for tissue spread) while repressing expression of protein A and proteases (Cheung *et al*., 2008).

Staphyloccocus aureus expresses other regulators that allow it to respond to environmental signals in addition to bacterial cell density (Yarwood and Schlievert, 2003). One of such is the *sae* (*S. aureus* exoprotein expression) which encodes the *SaeRS* two component system (Liu *et al*., 2016). *SaeRS* senses external stimuli and modulates virulence genes by binding to consensus sequences in promoter regions, thereby directly influencing their transcription (Balasubramanian *et al*., 2017). The *saeS*, i.e. the sensor histidine kinase, component of the *saeRS* system detects cognate environmental signals while the *SaeR* component directly upregulates virulence in response to these signals (Liu *et al*., 2016; Balasubramanian *et al*., 2017). Expression of the *sae* complex is regulated by environmental signals such as changes in pH, high concentrations of sodium chloride and subinhibitory levels of certain antibiotics (Balasubramanian *et al*., 2017). Furthermore,

exposure to phagocytosis-related signals such as hydrogen peroxide and antimicrobial peptides produced by neutrophils affect activity of the sae promoter (Balasubramanian *et al*., 2017).

1.6. Consequences of *S. aureus* binding and intracellular invasion

1.6.1. Internalisation

One confirmed mechanism employed by some pathogens to evade humoral immunity is to become internalised in the host cells (Bayles *et al*., 1998; Alva-Murillo *et al*., 2014). *Staphylococcus aureus* is generally not considered a significant intracellular pathogen of the magnitude associated with classic facultative intracellular pathogens (i.e. *Shigella* spp., *Mycobacterium, Listeria* and *Salmonella*) (Bayles *et al*., 1998; Menzies and Kourteva, 1998; Alexander *et al*., 2003). Traditionally, *S. aureus* has been regarded as a non-invasive extracellular pathogen that damages host cells after adhering to the extracellular matrix (Alexander *et al*., 2003). It is however now known and firmly established that *S. aureus* has the ability to infect and survive in various types of host cells, both non-professional phagocytic cells (NPPCs) and professional phagocytes (Balwit *et al*., 1994; Bayles *et al*., 1998; Bantel *et al*., 2001; Flannagan *et al*., 2016). Professional (or dedicated) phagocytic cells, such as macrophages and neutrophils, are equipped with receptor molecules that can detect foreign substances in the body. The primary purpose of their existence is phagocytosis (Lim *et al*., 2017). On the other hand, NPPCs, e.g. endothelial, epithelial, fibroblast, osteoblast, kidney cells, can also perform phagocytosis but, they have a more restricted set of targets (Lim *et al*., 2017). The ability of *S. aureus* to survive in the eukaryotic intracellular environment could explain several aspects of long-term colonisation and chronic staphylococcal diseases (Alexander *et al*., 2003) that are associated with multiple recurrences and which do not resolve even in the presence of what seems to be an adequate humoral immune response (Craigen *et al*., 1992; Greer and Rosenberg, 1993; Lowy, 1998).

Internalisation of *S. aureus* by NPPCs requires specific interaction between fibronectin and the fibronectin binding proteins (FnBPs) of *S. aureus* as well as host cell integrins (Alexander, 2001; Dziewanowska, *et al*., 1999; Krut *et al*., 2003). FnBPs, anchored in the bacterial cell wall, have multiple Fn-binding repeats in an unfolded region of the protein (Schwarz-Linek *et al*., 2003). Interaction of FnBP with Fn is mediated by tandem β zipper structures, via the binding of multiple Fn molecules by the repetitively arranged modules within a single FnBP (Schwarz-Linek *et al*., 2003; Fraunholz and Sinha, 2012). Consequently, FnBP-Fn sequester α5β1 integrins on the host cell surface (Fraunholz and Sinha, 2012). The high affinity and specificity of FnBPs for Fn is conferred by the tandem β-zipper structure they form together (Fowler *et al*., 2000; Schwarz-Linek *et al*., 2003). This adhesion and subsequent internalisation of *S. aureus* into NPPCs (mediated by a zipper-type mechanism) depends on the remodelling of the actin cytoskeleton and membrane dynamics of the NPPCs (DeMali *et al*., 2003; Hauck and Ohlsen, 2006; Fraunholz and Sinha, 2012). In 1999, Dziewanowska *et al*., demonstrated that this required actin polymerisation is dependent on tyrosine kinases.

Staphylococcus aureus expresses two closely related FnBPs, i.e. FnBPA and FnBPB, encoded by the genes *fnbA* and *fnbB* respectively (Alva-Murillo *et al*., 2014; Josse *et al*., 2017). Both have very similar domain organisations and sequences (Jonsson *et al*., 1991; Burke *et al*., 2010) but, their presence varies across the population (Peacock *et al*., 2000). In addition, both FnBPA and FnBPB consist of an amino-terminal secretion signal sequence followed by an A domain, that is closely related to fibrinogen-binding protein clumping factor A (ClfA) (Josse *et al*., 2017). However, the A domains of FnBPA and FnBPB only share 40% sequence identity (Burke *et al*., 2010). The A domain is followed by tandem repeats of Fn-binding regions (95% identity between FnBPA and FnBPB), 11 in FnBPA and 10 in FnBPB (Josse *et al*., 2017). This additional Fn-binding region, along with the higher Fn affinity of certain Fn-binding regions, might explain the higher overall affinity of FnBPA for Fn and the fact that FnBPA alone is sufficient for adhesion

and cell invasion (Grundmeier *et al*., 2004; Testoni *et al*., 2011). The importance of fibronectin binding proteins in the efficient uptake of *S. aureus* by NPPCs has been previously demonstrated in studies like the one by Fowler *et al*., 2000. Their study aimed to investigate the early stages of cellular invasion by *S. aureus* and also determine the bacterial and host components that are required for the process. They found that DU5883, an isogenic mutant of *S. aureus* NCTC 8325-4 defective in FnBP expression, cannot invade NPPCs. Heat shock protein 60 has previously been shown (in the study by Dziwanowska *et al*., 2000) to directly bind to FnBP exposed on the cellular surface. Findings from the study suggested that Hsp60 could function as a co-receptor with integrins linked through Fn.

Integrins, cation-dependent glycoprotein transmembrane receptors that are present on the surface of host cells, have an extracellular binding domain that recognises specific sequences (LVD and RGD) in fibronectin and other ligands (Kumar, 1998; Takada *et al*., 2007; Alva-Murillo *et al*., 2014); they contain non-covalently associated α- and β- subunits (Kumar, 1998) and at least 18 α- and 8 β-subunits have been described (Takada *et al*., 2007). These receptors mediate cellular adhesion and phagocytosis in addition to apoptosis, migration cancer and wound healing (Alva-Murillo *et al*., 2014). The β1 integrin has been shown to be necessary for efficient adhesion and endocytosis of *S. aureus* by non-professional phagocytes (Hoffmann *et al*., 2011). Blockage experiments with antibodies have been used to demonstrate the role of integrins during *S. aureus* internalisation into NPPCs (Alva-Murillo *et al*., 2014). Researchers like Sinha *et al*., 1999, Massey *et al*., 2001 and Kintarak *et al*., 2004, used specific antibodies to block integrin α5β1 in HEK 293, HUVEC cells and keratinocytes, respectively. Findings from their studies demonstrated that these receptors have a relevant role during *S. aureus* internalisation because their blockage led to a significant reduction of internalised bacteria. In 2012, Ridley *et al*. showed that both the availability and functional state of integrin α5β1 are crucial for *S. aureus* invasion in different epithelial cells.

Though the FnBP-Fn-α5β1 integrin pathway is widely acknowledged to be the main internalisation process, alternative mechanisms are involved in the adhesion and internalisation of *S. aureus* into host cells (Josse *et al*., 2017). Other mechanisms, mainly involving MSCRAMMs (such as bacterial serine aspartate repeat-containing protein D (*SdrD*), clumping factor A (ClfA), autolysin (Atl), and serine-rich adhesin for platelets (*SraP*)), are also used by *S. aureus* in a Fn-independent manner (Josse *et al*., 2017). However, it must not always be a surface-bound protein that triggers internalisation into host cells (Luqman *et al*., 2019). In 2018, Luqman *et al*., discovered a new mechanism of internalisation in mainly animal-pathogenic staphylococcal species. These species produce a subgroup of biogenic amines, the so-called trace amines (TAs) through the activity of staphylococcal aromatic amino acid decarboxylase (*SadA*). *SadA* decarboxylates aromatic amino acids to produce TAs, as well as dihydroxy phenylalanine and 5-hydroxytryptophan to thus produce the neurotransmitters dopamine and serotonin. Findings from the Luqman *et al*., 2018 study showed that *SadA*-expressing staphylococci showed increased adherence to HT-29 cells and 2- to 3-fold increased internalisation. They also saw that internalisation and adherence was increased in a *SadA* mutant in the presence of tryptamine. Furthermore, some internalisation mechanisms may support the FnBP-Fn-α5β1 integrin-mediated uptake of *S. aureus*; this is exemplified by *S. aureus* extracellular adherence protein (Eap) that plays a role in the adherence to fibroblasts and epithelial cells independently of any binding to Fn or fibrinogen (Harraghy *et al*., 2003; Josse *et al*., 2017).

1.6.2. Phagocytosis: Bacterial evasion strategies

The phagocytic process is usually very efficient and concludes with the destruction of the microorganism ingested (Uribe-Querol and Rosales, 2017). However, many successful pathogens (whether obligate intracellular pathogens or opportunistic) have evolved multiple strategies to prevent and/or inhibit phagocytosis (Smith and May, 2013; Uribe-Querol and Rosales, 2017). If

successful, pathogens are then able to lie dormant within a protected 'Trojan horse', disguised from the immune system (Smith and May, 2013).

One of such strategies is the prevention of phagocytosis i.e. prevention of ingestion by phagocytes (Urban *et al*., 2006; Uribe-Querol and Rosales, 2017). Some pathogens, including *S. aureus*, try to do that by producing substances that extracellularly intoxicate phagocytes (Flannagan *et al*., 2015; Uribe-Querol and Rosales, 2017). *Staphylococcus aureus* can secrete various membrane damaging toxins e.g. phenol soluble modulins, two-component leukotoxins and alpha toxin, that cause cell lysis and death (Flannagan *et al*., 2015; Teng *et al*., 2017). Furthermore, to prevent phagocytosis, microorganisms have evolved mechanisms to prevent opsonisation (Uribe-Querol and Rosales, 2017). *Staphylococcus aureus* can degrade opsonins by producing proteases such as staphylokinase (SAK) (Uribe-Querol and Rosales, 2017; Teng *et al*., 2017). SAK is a unique antiopsonic molecule that binds and activates surface-bound plasminogen into plasmin, which removes IgG as well as C3b from the bacterial surface (Teng *et al*., 2017). *Staphylococcus aureus* is also able to capture the opsonin so that it does not bind to the bacteria; this is well exemplified by the *S. aureus* protein A which specifically binds to the Fc region of IgG, preventing the antibody from engaging Fc receptors on phagocytes (Flannagan *et al*., 2015; Uribe-Querol and Rosales, 2017; Guerra *et al*., 2017; Teng *et al*., 2017).

Following phagocytosis, a series of coordinated fusion and fission events with specific compartments of the endocytic pathway ultimately leads to the generation of a phagolysosome (Mitchell *et al*., 2016); this process is known as phagosomal maturation (Flannagan *et al*., 2009). The phagolysosome possesses potent microbicidal features, and has a lumen that constitutes a highly acidic, oxidative, and degradative environment (Mitchell *et al*., 2016). Although the phagolysosomal lumen is an inhospitable environment, many successful pathogens perturb host cell function to impede or alter conventional phagosome maturation (Flannagan *et al*., 2015).

These pathogens present intracellular survival strategies such as inhibition of phagosome-lysosome fusion, survival inside the phagolysosome and escape into the cytoplasm (Urban *et al*., 2006). They execute these survival strategies by blocking different points during phagosome maturation i.e. acidification of the phagosome, reducing activation of the NADPH oxidase, and preventing phagosome to lysosome fusion (Uribe-Querol and Rosales, 2017).

During maturation, the pH of phagosomes quickly drops from neutral to 5, through a high activity of a vesicular proton-pump ATPase (H+ V-ATPase) (Flannagan *et al*., 2015; Queval *et al*., 2017). An acidic pH is essential for the optimal activity of lysosomal digestive enzymes and for reactive oxygen species production; therefore, phagosomal acidification is a prerequisite for intracellular bacterial clearance (Queval *et al*., 2017). *Mycobacterium tuberculosis* inhibits phagosome maturation by suppressing phagosomal acidification and then persists in the relatively lower acidic environment (pH ~6.2) (Zhai *et al*., 2019). Acidification of *M. tuberculosis* phagosomes is inhibited by preventing the accumulation of H+ V-ATPase on the phagosome membrane (Sturgill-Koszycki *et al*., 1994; Smith and May, 2013). *Mycobacterium tuberculosis* phagosomes also avoid lysosome fusion by disrupting host Ca^{2+} signalling (Flannagan, 2009; Smith and May, 2013).

Pathogens also possess various mechanisms to resist the antimicrobial components found in the phagolysosomal lumen (Uribe-Querol and Rosales, 2017). One of such pathogens is *S. aureus* that can resist the lytic effect of lysozyme on the cell wall peptidoglycan due to its ability to express the enzyme O-acetyltransferase A (which causes O-acetylation of the peptidoglycan) (Bera *et al*., 2005; Flannagan *et al*., 2015). Microbes including *S. aureus*, *Helicobacter pylori*, *Cryptococcus neoformans* and *Coccidioides posadasii* express urease, an enzyme that catalyses the hydrolysis of urea to form carbon dioxide and ammonia, resulting in the pH neutralisation of the phagosome (Uribe-Querol and Rosales, 2017; Fu *et al*., 2018; Zhou *et al*., 2019). Some pathogens have also evolved ways to overcome the very damaging oxidative environment of the phagolysosome i.e.

the effects of reactive oxygen and reactive nitrogen species (Flannagan *et al*., 2009; Uribe-Querol and Roslaes, 2017). For instance, *S. aureus* can express five types of enzymes or pigment that promote resistance to oxidative killing i.e. superoxide dismutase, methionine sulfoxide reductases, catalase, adenosine synthase A and staphyloxanthin, (Teng *et al*., 2017).

The phagolysosome is a place where microbial nutrients are eliminated to arrest pathogen growth; in response, several microorganisms have evolved mechanisms to retain these important nutrients (Flannagan *et al*., 2015; Uribe-Querol and Rosales, 2017). Divalent cations, such as Fe^{2+}, Zn^{2+} and Mn^{2+}, are actively transported out of the phagolysosome (Cellier *et al*., 2007). However, pathogens such as *S. aureus* and *M. tuberculosis* produce siderophores, which are low-molecular weight Fe^{2+}-binding molecules of extremely high affinity, that remove Fe^{2+} from host proteins, such as haemoglobin, and transferrin (Serafini *et al*., 2009; Beasley *et al*., 2011). These siderophores can trap enough Fe^{2+} to allow bacterial survival (Uribe-Querol and Rosales, 2017).

Several pathogens, e.g. *Cryptococcus neoformans*, *Listeria monocytogenes*, and *M. tuberculosis*, also have the capacity to completely escape the phagolysosome (Uribe-Querol and Rosales, 2017). Vomocytosis is a non-lytic mechanism that allows the phagocytosed pathogen, e.g. *C. neoformans*, to escape the phagocytic cell alive (Alvarez and Casadevall, 2006; Smith and May, 2013). The process involves an exocytic fusion of the phagosome with the plasma membrane, thus releasing the pathogen (Johnston and May, 2010). *Listeria monocytogenes* uses listeriolysin O (LLO), a pore-forming toxin that permeabilises the phagosome membrane, to escape the phagosome (Flannagan *et al*., 2009; Smith and May, 2013; Queval *et al*., 2017). *Staphylococcus aureus* is also able to escape the phagolysosome in an *agr* dependent process that precedes host cell death (Fraunholz and Sinha, 2012; Giese *et al*., 2011; Darisipudi *et al*., 2018). The pore-forming staphylococcal α-toxin is known to mediate this escape; however, it is now known that the α-toxin alone is not sufficient to mediate phagolysosomal escape (Giese *et al*., 2009; Giese *et al*., 2011).

Phenol soluble modulins, produced by *S. aureus* upon internalisation by phagocytes, help *S. aureus* escape from the phagolysosome, invade the cytoplasm and possibly kill the host cell (Darisipudi *et al*., 2018).

1.6.3. Apoptosis and Necrosis

Two main outcomes of host cell invasion by *S. aureus* are generally described: (a) rapid host cell lysis, triggered by the secretion of toxins and other pro-inflammatory factors, which induce strong inflammatory and cytotoxic effects (Proctor *et al*., 2014; Rollin *et al*., 2017); (b) persistence within morphologically-intact host cells for extended periods of time, where secretion of virulence factors is either down-regulated or not expressed (Proctor *et al*., 2014; Strobel *et al*., 2016; Rollin *et al*., 2017). The fates of the pathogen and the infected host cell depend on the staphylococcal isolate and genotype, as well as differential susceptibility of host cells to virulence factors, host cell gene expression, etc. (Krut *et al*., 2003; Fraunholz and Sinha, 2012; Strobel *et al*., 2016).

Findings from studies like those by Bayles *et al*., 1998, Menzies and Kourteva, 1998, Wesson *et al*., 2000, Kahl *et al*., 2000, Nuzzo *et al*., 2000, Tucker *et al*., 2000, Haslinger *et al*., 2003, Genestier *et al*., 2005, have shown that when virulent *S. aureus* strains are added to host cells in tissue culture, host cell death occurs via mechanisms that have been mainly identified as apoptotic. *Staphylococcus aureus*-mediated apoptosis may cause tissue damage, compromise the antimicrobial immune response thereby promoting bacterial spread (Loffler *et al*., 2005). Furthermore, the induction of apoptosis in endothelial cells might play a pivotal role in microbial pathogenesis by providing a route for dissemination of *S. aureus* to distant sites where foci of metastatic infection could be established (Loffler *et al*., 2008). The virulence factors required for *S. aureus*-induced apoptosis in epithelial cells depend on *agr* and the alternative stress–response sigma-factor σB, but mainly seem to be independent of *SarA* (Fraunholz and Sinha, 2012).

Several studies have shown that expression of the α-toxin is both required and sufficient for *S. aureus* induced cell death, either apoptotic or necrotic (Menzies and Kourteva, 2000; Bantel *et al*., 2001; Essmann *et al*., 2003; Haslinger *et al*., 2003). Bantel *et al*., in their 2001 study to investigate the mechanism of cell death in T cell after *S. aureus* infection, noted that bacterial invasion is not required and that soluble α-toxin is a mediator of *S. aureus*–induced apoptosis. In contrast to this, researchers like Bayles *et al*., 1998, Menzies and Kourteva, 1998, and Tucker *et al*., 2000, had previously proposed that *S. aureus*-induced apoptosis required the prior internalisation of bacteria (epithelial cells, endothelial cells and osteoblasts, respectively, were used in these studies). This contradiction in findings may reflect a cell-type-specific phenomenon as some cell types, e.g. lymphocytes, are known to be especially sensitive to killing by α-toxin (Krut *et al*., 2003; Fraunholz and Sinha, 2012). Further supporting the possibility of a cell-type-specific phenomenon being responsible for the contradiction in the study outcomes is the fact that although human endothelial cells are virtually insensitive to the action of *S. aureus* α-toxin, comparatively low numbers of *S. aureus* cells with a combined invasive and strongly haemolytic phenotype readily induce apoptotic cell death in HUVEC (Haslinger-Loffler *et al*., 2005; Fraunholz and Sinha, 2012). Furthermore, differences in experimental approaches employed by the researchers may also be responsible for the contradictions. For instance, in the Bantel *et al*., 2001 study, supernatants of 14-hr *S. aureus* cultures were used, which were stationary, well above quorum sensing, and which, though filtered, probably contained significant amounts of toxic molecules derived from dead bacteria (Krut *et al*., 2003). At low doses, α-toxin binds to specific, cell surface receptors to produce small heptameric pores that selectively facilitate the release of monovalent ions, resulting in DNA fragmentation and apoptosis (Jonas *et al*., 1994; Song *et al*., 1996; Valeva *et al*., 1997). In addition, *S. aureus* α-toxin non-specifically adsorbs to the lipid bilayer of the host cell membrane, forming larger pores that are Ca^{2+} permissive, which results in massive necrosis (Essmann *et al*., 2003).

Other virulence factors expressed by *S. aureus* are also known to induce apoptosis in host cells. Findings from the study by Genestier *et al*., 2005 showed that PVL induces polymorphonuclear cell death (by apoptosis or necrosis) in a concentration dependent manner. Findings from this study further showed that PVL-induced apoptosis was associated with a rapid disruption of mitochondrial homeostasis and activation of caspase-9 and caspase-3, which suggested that that PVL-induced apoptosis is preferentially mediated by the mitochondrial pathway. Loffler *et al*., 2010, further demonstrated that PVL induces rapid activation and cell death of neutrophils in a species-specific manner, i.e. cell death was induced in human and rabbit neutrophils, but not in murine or simian cells.

Based on macroscopic morphological alterations manifested during cell death, as well as mechanisms whereby dead cells and their fragments are disposed of, apoptosis has historically been classified as cell death (type I) exhibiting features such as cytoplasmic shrinkage, chromatin condensation (pyknosis), nuclear fragmentation (karyorrhexis), and plasma membrane blebbing, culminating with the formation of apparently intact small vesicles (commonly known as apoptotic bodies) that are efficiently taken up by neighbouring cells with phagocytic activity and degraded within lysosomes (Schweichel *et al*., 1973; Galluzzi *et al*., 2007; Green *et al*., 2012; Galluzzi *et al*., 2018). Furthermore, apoptosis has also been defined as cell death accompanied by the activation of caspase proteases (Galluzzi *et al*. 2012). However, based on the updated classification of cell death proposed by Galluzzi *et al*., 2018, apoptosis can be either intrinsic or extrinsic. Intrinsic apoptosis is defined as a form of regulated cell death (RCD) initiated by a variety of micro-environmental perturbations including (but not limited to) reactive oxygen species overload, growth factor withdrawal, endoplasmic reticulum stress, replication stress, DNA damage, microtubular alterations or mitotic defects (Nunez *et al*., 1990; Czabotar *et al*., 2014; Roos *et al*., 2016; Pihan *et al*., 2017; Vitale *et al*., 2017). On the other hand, extrinsic apoptosis is defined as a form of RCD that is initiated by perturbations of the extracellular environment (Ashkenazi *et al*.,

1998; Flusberg *et al*., 2015; Gibert *et al*., 2015). Extrinsic apoptosis is mostly driven by either of two types of plasma membrane receptors i.e. death receptors (whose activation depends on the binding of the cognate ligand(s)), or dependence receptors (whose activation occurs when the levels of their specific ligand drop below a specific threshold) (Aggarwal *et al*., 2012; Gibert *et al*., 2017). Contrary to what was previously thought, both intrinsic and extrinsic apoptosis and consequent efferocytosis (mechanism whereby dead cells and fragments thereof are taken up by phagocytes and disposed) are not always immunologically silent (Green *et al*., 2009; Green *et al*., 2016; Yatim *et al*., 2017). The process of apoptosis is essentially mediated by caspases, a family of intracellular cysteine proteases, which cleave a variety of cellular proteins leading to the final demise of the cell (Los *et al*., 1999; Fischer *et al*., 2003; Fraunholz and Sinha, 2012).

Based on macroscopic morphological alterations manifested during cell death, necrosis has historically been classified as cell death (type III) displaying no distinctive features of apoptosis or autophagy and terminating with the disposal of cell corpses in the absence of obvious phagocytic and lysosomal involvement (Schweichel *et al*., 1973; Galluzzi *et al*., 2007; Galluzzi *et al*., 2018). Autophagy (type II cell death) is a catabolic process engaged under metabolic stress (such as nutrient starvation and bioenergetics failure), to ensure availability of critical metabolic intermediates (Green *et al*., 2015). It is also important for the removal of damaged organelles (including mitochondria), protein aggregates, and infecting organisms (Levine and Kroemer, 2008; Kroemer *et al*., 2010; Green *et al*., 2015). Necrotic cell death encompasses a wide variety of cell death processes with one common denominator, i.e. the loss of plasma membrane integrity followed by cytoplasmic leakage (Yuan and Kroemer 2010; Green *et al*., 2015). Although necrosis can occur as a consequence of irreparable cell damage, at least one pathway of active necrosis exists (Green *et al*., 2015). This form of cell death, sometimes called necroptosis, is engaged by several signalling pathways that all converge on the activation of receptor interacting protein kinase 3 (Green *et al*., 2015; Galluzzi *et al*., 2018). It is now clear that necroptosis not only

mediates adaptative functions upon failing responses to stress, but also participates in developmental safeguard programs (to ensure the elimination of potentially defective organisms before parturition), as well as in the maintenance of adult T-cell homeostasis (Galluzzi *et al*., 2018).

In some instances, cell death is passive and does not require the activation of any particular signalling pathway; in such cases, necrosis could occur simply as a consequence of extensive damage that results in disruption of cell integrity, e.g. at high temperature, following freeze-thaw, or upon mechanical stress (Green *et al*., 2015). Furthermore, a necrotic morphology (i.e., rupture of the plasma membrane) can also be observed at late stages of an apoptotic or autophagic cell death program, when dead cells fail to be cleared from the system by efferocytosis (Green *et al*., 2015). This is referred to as secondary necrosis, and it is independent of any other signalling event than those initially engaged (apoptotic or autophagic) (Green *et al*., 2015).

1.7. Infections caused by *S. aureus*

1.7.1. Bacteraemia/Sepsis: Bacteraemia indicates the presence of viable bacteria in the circulatory blood and is usually defined clinically as positive blood cultures (Curry and Lewis, 2003). *Staphylococcus aureus* is the second leading cause of bacteraemia (Salgado-Pabon *et al*., 2013). An increasing population with underlying conditions (e.g. renal haemodialysis, diabetes mellitus, liver disease, immunosuppression) and medical advances have contributed to the surge of *S. aureus* infections (Salgado-Pabon *et al*., 2013); therefore, it is not surprising that the incidence of *S. aureus* bacteraemia (SAB) may be increasing, in some regions, due to higher numbers of invasive procedures and/or at-risk situations, including the presence of endovascular devices (Le-Moing *et al*., 2015). SAB often results from skin infections, surgical wounds, pneumonia, infected catheters or intravenous drug use and is associated with a mortality rate of 20 to 40% (Salgado-Pabon *et al*., 2013). The site of infection, patients' co-morbidities as well as methicillin-resistance

affect SAB acquisition as well as outcome (Bassetti *et al*., 2017). SAB may be associated with a deep infectious focus (whether cause or consequence of bacteraemia e.g. bone and joint, soft tissue, central nervous system, infective endocarditis (IE) and intra-abdominal/pelvic infections) or without any detected deep focus of infection (Le-Moing *et al*., 2015). SAB associated with IE are the most frequent and have the poorest prognosis whether or not it (IE) is a consequence of SAB (Le-Moing *et al*., 2015).

Invasive *S. aureus* infections are frequently associated with bacteraemia which may rapidly advance to sepsis (McAdow *et al*., 2011). Sepsis is defined as a systemic inflammatory response syndrome (SIRS) in response to severe infection; it is one of the major causes of death in the world, in particular developing and underdeveloped countries (Alavi *et al*., 2014). It is associated with high mortality because of continued activation and apoptosis of immune cells and malperfusion (loss of blood supply) of organ systems (McAdow *et al*., 2011). Dysregulation of the physiological coordination between haemostasis and inflammation which triggers intravascular fibrin deposits (disseminated intravascular coagulation-DIC) occurs during sepsis; this dysregulated clotting places those with sepsis at a high risk for systemic bleeding and loss of perfusion for vital organ systems (McAdow *et al*., 2011).

1.7.2. Infective endocarditis: IE is an infection of the heart endothelium, predominantly valves, that results in the formation of large vegetative lesions (i.e. a mesh-work of host factors, such as fibrin/platelets, and bacterial aggregates) (Salgado-Pabon *et al*., 2013). *Staphylococcus aureus* is the predominant causative agent of IE (Salgado-Pabon *et al*., 2013; Bouchiat *et al*., 2015). *Staphylococcus aureus* infective endocarditis (SAIE) is mostly acquired in the hospital and intravascular devices are often the source of infection (Naber, 2009). It accounts for up to one-third of SAB complications and is associated with high risk for metastatic abscesses, persistent bacteraemia, congestive heart failure and systematic embolisation, all of which can lead to death

(Salgado-Pabon *et al*., 2013). Outcomes of SAIE (particularly those due to MRSA) tend to be worse than IE caused by other bacterial pathogens (Naber, 2009). Drug use, congenital heart disease, or the presence of cardiac prosthetic material have been identified as host-related IE risk factors; however, the fact that about 30-50% of IE cases occur without any described host-risk factors points to the involvement of bacterial features in the occurrence of IE during bacteraemia (Bouchiat *et al*., 2015). Virulence factors expressed by *S. aureus*, particularly those involved in tissue adherence/colonisation (coagulases, von Willebrand factor binding protein, clumping factor ClfA) and those involved in survival in the bloodstream (SOK, a surface factor that promotes resistance to oxidative/neutrophil killing) are associated with the pathogenesis of IE (Salgado-Pabon *et al*., 2013).

1.7.3. Pneumonia: Pneumonia is an acute form of respiratory tract infections in which the lung alveoli becomes filled with fluid thereby causing painful breathing and limiting intake of oxygen (Roomaney *et al*., 2016). It can affect people of all ages however, children under 5 years, immunocompromised individuals and adults over 65 years tend to be more at risk (Roomaney *et al*., 2016). Pneumonia can be acquired both in the community and hospital environments and can be transmitted by inhalation or aspiration of a pathogenic microorganism (Cilloniz *et al*., 2016). Pneumonia caused by *S. aureus* constitutes 1-10% of cases of community-acquired pneumonia and up to 20-50% of cases of nosocomial pneumonias (Kreienbuehl *et al*., 2011). Furthermore, approximately 50% of the patients with pneumonia caused by *S. aureus* have underlying co-morbidities and risk factors (Kreienbuehl *et al*., 2011). An association between necrotising pneumonia and PVL-producing *S. aureus* was established in 1999 (Gillet *et al*., 2002). Clinical manifestations of this type of pneumonia do not seem to differ from those of pneumonia due to other bacterial pathogens (Gonzalez *et al*., 1999; Gonzalez *et al*., 2003).

1.7.4. Osteoarticular infections

1.7.4.1. Arthritis: Bacterial arthritis (septic arthritis) is a rapidly progressive and highly destructive joint disease in humans (Hultgren *et al*., 1998). Bacterial replication in the joint and the ensuing inflammatory process can lead to rapid local joint destruction which may be accompanied by systemic infection (Shariff *et al*., 2013). The most common predisposing factor for septic arthritis is rheumatoid arthritis (RA), which is present in 10-40% cases of septic arthritis (Hultgren *et al*., 1998). This could be because the abnormal joint architecture in rheumatoid arthritis may allow microorganisms to escape normal phagocytosis or that patients with RA may have reduced bactericidal activity of synovial fluid and defective phagocytosis by polymorphonuclear cells (Shariff *et al*., 2013). The use of disease-modifying anti-rheumatic drugs also increases the risk of septic arthritis (Shariff *et al*., 2013).

1.7.4.2. Osteomyelitis: Is an infection of the bone resulting in its inflammatory destruction, bone necrosis, and new bone formation (Tong *et al*., 2015). It is typically of bacterial origin, with *S. aureus* being the most prevalent pathogen (Josse *et al*., 2015). In children, osteomyelitis is mostly a result of haematogenous spread from distant infected tissues; however, surgical procedures are becoming increasingly responsible for a direct contamination of bone tissue, especially in the presence of medical devices, in adults (Josse *et al*., 2015). Osteomyelitis is devastating for many patients with long-term consequences due to the challenges associated with treatment (Olson and Horswill, 2013). Factors that affect treatment include poor bioavailability of antibiotics in bone tissue, rising antibiotic resistance and the biofilm-like properties of the infection (Olson and Horswill, 2013). As such osteomyelitis is often not treatable with antibiotics (Corrado *et al*., 2016) with surgical debridement to remove the invading bacteria and removal/replacement of infected prosthetic joint being the only options available to physicians (Olson and Horswill, 2013).

1.7.5. Skin and soft tissue infections (SSTIs): SSTIs are ubiquitous infections that reflect inflammatory microbial invasion of the epidermis, dermis and subcutaneous tissues (Dryden, 2010). They range in severity from minor, self-limiting and superficial infections to life-threatening diseases (Roberts and Chambers, 2005; Gu *et al*., 2015). According to the practice guidelines of the Infectious Diseases Society of America (IDSA) for the diagnosis and management of SSTIs, they can be classed as superficial, uncomplicated infections (e.g. cellulitis, erysipelas and impetigo), necrotising infection, surgical site infections, infections associated with bites and animal contact and infections in immunocompromised host (Dryden, 2010). Classic signs of inflammation in SSTIs are heat, redness, swelling, pain and discharge (Dryden, 2010). *Staphylococcus aureus* is the leading cause of SSTIs across all continents (Gu *et al*., 2015). Primary pyodermas (such as carbuncles, folliculitis, furuncles and impetigo) are examples of skin infections caused by *S. aureus*; moreover, infections involving the soft tissues, such as pyomyositis and cellulitis, are less common but can cause serious morbidity (Roberts and Chambers, 2005).

1.7.6. Surgical site infections (SSIs): SSIs are infections that occur at incision site within 30 days after surgery (Chaudhary *et al*., 2017). They are among the most common hospital-acquired infections and constitute an important quality criterion in health research (Mellinghoff *et al*., 2018). SSIs are associated with poor outcome (Mellinghoff *et al*., 2018) and patients with SSIs are at 2-11 times greater risk of death compared to patients without SSI (Tariq *et al*., 2017). As confirmed by the European Centre for Disease Prevention and Control (ECDC), *S. aureus* is the most common cause of SSIs (Mellinghoff *et al*., 2018). SSI due to *S. aureus* are due to endogenous microbes; molecular epidemiology has demonstrated that the strain of *S. aureus* that causes post-operative infection is identical to the strain isolated from the nasal cavity pre-operatively in 85% of patients (Kontra, 2012). Patient related factors, such as obesity, anaemia, malnutrition, including factors like duration of surgery, pre-operative and prolonged post-operative hospital stay and poor post-operative glycaemic control are risk factors that predispose to SSIs (Tariq *et al*., 2017).

1.8. Immune response to *S. aureus* infection

Immune response against *S. aureus* involves activation of both the innate and adaptive immune systems (Karauzum and Datta, 2017). First, *S. aureus* overcomes the physical protective barriers of the human body through invasion of soft tissues, surgical wounds, or medical devices to establish a focus of infection (Wardenburg *et al*., 2006; Cheng *et al*., 2009). Following infection, the innate immune response, which is the first line of defence, is activated by pattern recognition pathways that detect non-specific markers of microbial infection (Karauzum and Datta, 2017). This results in the activation of phagocytic cells e.g. neutrophils and macrophages (Karauzum and Datta, 2017). Clearance of *S. aureus* from the bloodstream results from the phagocytic killing by the effector cells of the innate immune system, i.e. neutrophils and macrophages, and by opsonising antibodies directed against capsule and peptidoglycan (Deshmukh *et al*., 2009). Binding of bacteria to host organ tissues also brings about clearance of *S. aureus* from the bloodstream (Wardenburg *et al*., 2006). Those staphylococci that escaped killing replicate in infected tissues and generate a proinflammatory response mediated by the release of chemokines and cytokines, neutrophils, macrophages and other cells of the innate immune system (Wardenburg *et al*., 2006; Deshmukh *et al*., 2009). The responsive invasion of immune cells to the site of infection is accompanied by liquefaction necrosis as the host seeks to prevent staphylococcal spread and allow for removal of necrotic tissue debris (Cheng *et al*., 2009). Early and effective launch of the innate immune response limits the establishment of infectious foci, thereby curbing the severity of staphylococcal infections (Wardenburg *et al*., 2006; Deshmukh *et al*., 2009). Following these events, the adaptive immune response kicks in, during which time T and B cells capable of specific antigen recognition lead to the eradication of staphylococci (Wardenburg *et al*., 2006). In addition to having direct activity against bacteria, T cells and antibodies also amplify the activity of innate immune cells (Karauzum and Datta, 2017). Thus, the coordinated action of the innate and adaptive immune response is critical for efficient pathogen

elimination (Wardenburg *et al*., 2006). Although the relative paucity of systemic infections despite the high rate of *S. aureus* colonisation is evidence of the protective role played by the adaptive immune response, prevalence of recurrent infections with *S. aureus* suggest that the adaptive immune response is not completely effective (Karauzum and Datta, 2017).

1.9. Antibiotic classes used in the treatment of suspected or confirmed cases of *S. aureus* infections

1.9.1. β-lactams: β-lactams are antibiotics that have a four-membered β-lactam ring in their molecular structure (Calderon and Sabundayo, 2007; Ullah and Ali, 2017; Bozcal and Dagdeviren, 2017). This β-lactam ring is essentially a highly reactive 3-carbon and 1-nitrogen ring (Etebu and Arikekpar, 2016) (as shown in Figure 1.1). Variation in the side chain or additional ring(s) differentiates the different classes of β-lactam antibiotics (Ullah and Ali, 2017).

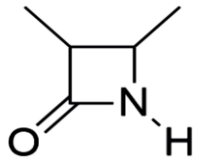


Figure 1.1: Basic structure of the *β*-lactam ring (Ullah and Ali, 2017)

The activity of the β-lactam antibiotics depends on their affinity for penicillin-sensitive enzymes i.e. the penicillin-binding proteins (Denyer *et al*., 2004). β-lactams can covalently bind penicillin-binding proteins (PBPs), which are responsible for cross-linking peptide units during peptidoglycan synthesis; by so doing, they interfere with the synthesis of the bacterial cell wall thus resulting in lysis and cell death (Etebu and Arikekpar, 2016; Bozcal and Dagdeviren, 2017). As the antibacterial activity of β-lactams resides in the β-lactam ring, cleavage of the ring by bacterial β-lactamases/penicillinases inactivates the compound, rendering them ineffective (Calderon and Sabundayo, 2007). Though resistance results more commonly from β-lactamases

(Denyer *et al*., 2004), decreased ability to penetrate the cell wall and reach the PBP as well as decreased affinity of the PBP to the antibiotic could also bring about resistance to β-lactams (Calderon and Sabundayo, 2007). β-lactams have been demonstrated to exhibit time-dependent killing to *S. aureus in vitro*; therefore, optimisation of the duration of exposure to the drug should be the goal of a dosing regimen for β-lactams that are active against *S. aureus* (Rayner and Munckhof, 2005).

1.9.1.1. Penicillins: They are β-lactam antibiotics that contain a 6-animopenicillanic acid (lactam plus thiazolidine/penam ring) nucleus and a side chain (Calderon and Sabundayo, 2007; Etebu and Arikekpar, 2016) (as shown in Figure 1.3).

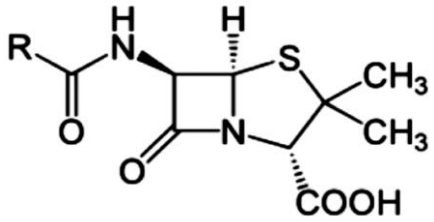


Figure 1.2: Core structure of penicillins (penam skeleton); R represents side chains that differ among penicillins (Terico and Gallagher, 2014)

Penicillins can be either naturally occurring, e.g. benzylpenicillin/penicillin G, phenoxymethyl penicillin (penicillin V), or semi-synthetic (Denyer *et al*., 2004) e.g. the aminopenicillins, penicillinase-resistant penicillins, carboxypenicillins and the ureidopenicillins (Calderon and Sabundayo, 2007). Natural penicillins were once considered the drug of choice for infections caused by non-β-lactamase producing staphylococci, however, clinical use is limited due to widespread resistance (Calderon and Sabundayo, 2007). Emergence of penicillinase-producing staphylococci led to the development of the penicillinase-resistant penicillins that had an additional side chain which protected the β-lactam ring from the action of penicillinases; examples include methicillin, nafcillin, oxacillin (Calderon and Sabundayo, 2007). Penicillins, such as ticarcillin,

amoxicillin and piperacillin, are only active against the small percentage of *S. aureus* strains that do not produce penicillinase (Etebu and Arikekpar, 2016). Penicillinase-resistant penicillins have excellent activity against *Staphylococcus* species (including strains of penicillinase-producing *S. aureus*). All penicillins are bactericidal (Calderon and Sabundayo, 2007). Though usually nontoxic at clinically effective levels, the major side effect associated the use of penicillin is hypersensitivity (Levinson and Jawets, 2002). They are the antimicrobial agents most commonly used to treat serious infections caused by methicillin susceptible *S. aureus* (MSSA) i.e. strains of *S. aureus* that do not produce penicillinase (Rayner and Munckhof, 2005; Calderon and Sabundayo, 2007).

1.9.1.2. Cephalosporins: Consist of a six-membered dihydrothiazine ring fused to a β-lactam ring (Denyer *et al*., 2004) and are substituted in two places on the 7-aminocephalosporanic acid nucleus (Levinson and Jawets, 2002) (as shown in Figure 1.3). Cephalosporins are thus similar to penicillins in both mode of action and structure (Denyer *et al*., 2004; Etebu and Arikekpar, 2016). They have side chains that enable them attach to different PBPs and resist breakdown by penicillinase producing bacterial strains (Etebu and Arikekpar, 2016).

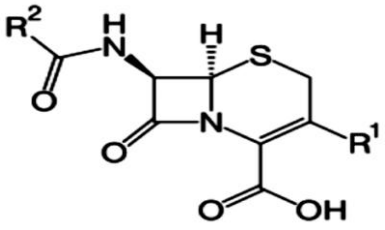


Figure 1.3: Core structure of cephalosporins; R^1 and R^2 represent side chains that differ among cephalosporins (Terico and Gallagher 2014)

Though bactericidal (Calderon and Sabundayo, 2007), cephalosporins are not as bactericidal against *S. aureus in vitro* as penicillins (Rayner and Munckhof, 2005). Due to the fact that there is potentially less toxicity than penicillinase-resistant penicillins and the fact that dosing is more convenient, cephalosporins are indicated in the treatment of less severe staphylococcal infections

such as skin and soft tissue infections (Rayner and Munckhof, 2005). Cephalosporins have traditionally been divided into groups/generations based on their spectrum of activity:

(i) First generation cephalosporins, e.g. cefazolin, which have good activity against Gram positive bacteria and relatively modest activity against Gram negative bacteria (Pacifici, 2011);

(ii) Second generation cephalosporins, e.g. cefoxitin, which have increased activity against Gram negative microorganisms (Pacifici, 2011). However, most second generation cephalosporins display moderate activity against *Streptococcus* and *Staphylococcus* (Chaudhry, 2019);

(iii) Third generation cephalosporins, e.g. cefixime, are the first generation to be considered extended-spectrum cephalosporins; they are more stable to common β-lactamases produced by Gram-negative bacilli but are hydrolysed by broad-spectrum β-lactamases, such as extended-spectrum β-lactamases, AmpC-producing organisms, and carbapenemases among others (Chaudhry, 2019). They have even less staphylococcal activity compared to the first and second generation cephalosporins (Calderon and Sabundayo, 2007);

(iv) Fourth generation cephalosporins, e.g. cefepime, which were designed to maintain good activity against both Gram positive and negative organisms (Calderon and Sabundayo, 2007). They are particularly useful for the empirical treatment of serious infections in hospitalised patients when Enterobacteriaceae and *Pseudomonas* are potential aetiologies (Pacifici, 2011); and,

(v) Fifth generation cephalosporins, otherwise known as anti- MRSA cephalosporins, e.g. ceftaroline and ceftibiprole; they offer good coverage against Gram positive cocci (e.g., MSSA, MRSA, and *Streptococcus* spp.) and enteric Gram negative rods, with the

exception of extended-spectrum beta-lactamase producers *Acinetobacter baumanii* and *Stenotrophomonas maltophilia* (Chaudhry *et al*., 2019).

Ceftolozane/tazobactam, one of the newer cephalosporins, has not yet been categorised into an existing generation because of its unique spectrum of activity (Chaudhry *et al*., 2019). Nevertheless, it has been shown to have good coverage against enteric Gram-negative bacilli, *P. aeruginosa*, and *Streptococcus* spp. (Chaudhry *et al*., 2019). Cephalosporins can also be classified into four groups on the basis of microbiology and pharmacology, i.e. cephalosporins with

(i) Increased activity against Gram-positive bacteria: cephalothin, cefazolin;

(ii) Increased activity against enterobacteria and parvobacteria

   a. Cefuroxime, cefamandole

   b. Cefotaxime, ceftriaxone

   c. Cefepime, cefpirome;

(iii) Increased activity against *Enterobacteria, Parvobacteria* and *Pseudomonas spp*: ceftazidime, cefoperazone;

(iv) Increased activity against *Enterobacteria* and *Bactericides*: cefoxitin, cefotetan.

(Shahbaz *et al*., 2017).

1.9.1.3. Carbapenems: They are made up of a β-lactam ring attached to a 5-membered ring (Terico and Gallagher, 2014) (as shown in Figure 1.4).

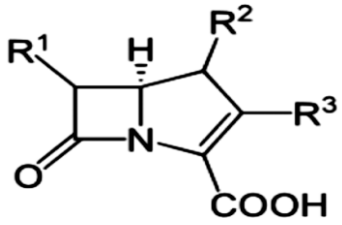


Figure 1.4: Basic structure of carbapenems; R^1, R^2 and R^3 represent side chains that differ among carbapenems (Terico and Gallagher, 2014)

Carbapenems are extremely broad-spectrum, parenteral β-lactam agents (Rayner and Munckhof, 2005) that are active against most strains of MSSA (Calderon and Sabundayo, 2007). Though they can be used to treat MSSA in mixed infection, it is recommended that they be held in reserve for the treatment of staphylococcal infections (Rayner and Munckhof, 2005). In other words, carbapenems should only be administered when patients with infection become gravely ill or are suspected of harbouring resistant bacteria (Etebu and Arikekpar, 2016). Imipenem (has the widest spectrum of activity of the β-lactam drugs) (Levinson and Jawetz, 2002); ertapenem and meropenem are examples of carbapenems (Rayner and Munckhof, 2005; Etebu and Arikekpar, 2016).

1.9.2. Macrolides: Are composed of a minimum of two amino and/or neutral sugars attached to a lactone ring of variable size (Fong and Drlica, 2008) (exemplified in Figure 1.5).

Erythromycin **Azithromycin**

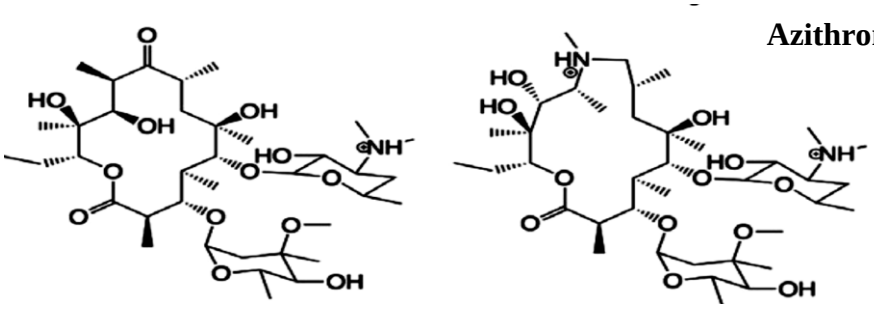


Figure 1.5: Structures of some 14-membered macrolide antibiotics (Pavlova *et al*., 2017)

They inhibit protein synthesis by binding to the 50S ribosome thus preventing addition of amino acids to the polypeptide chain during protein synthesis (Rayner and Munckhof, 2005; Etebu and Arikekpar, 2016). Macrolides are broad-spectrum antibiotics and their spectrum of activity is wider than that of penicillins and they are often administered to patients allergic to penicillin (Etebu and Arikekpar, 2016). However, macrolides are bacteriostatic against extracellular pathogens including *S. aureus* (Rayner and Munckhof, 2005). Examples include erythromycin and clarithromycin (Rayner and Munckhof, 2005). Resistance to macrolides could result from target

modification, mediated by an rRNA *erm* methylase that alters a site in 23S rRNA of the 50S ribosomal subunit common to the binding of macrolides, lincosamides and streptogramin B (Schmitz *et al*., 1999). This modification of the ribosomal target confers cross-resistance to macrolides, lincosamides and streptogramin B antibiotics (MLS_B resistant phenotype) (Schmitz *et al*., 1999). The presence of multi-component macrolide efflux pumps (*msrA and msrB*) as well as enzymatic inactivation of macrolides (enzymes EreA and EreB) have also been documented in staphylococci (Schmitz *et al*., 1999). Common side effects associated with the use of macrolides involve the GI tract e.g. vomiting, nausea, diarrhoea and abdominal pain (Calderon and Sabundayo, 2007).

1.9.3. Tetracyclines: Are antibiotics with four cyclic rings (Levinson and Jawetz, 2002) (as shown in Figure 1.6).

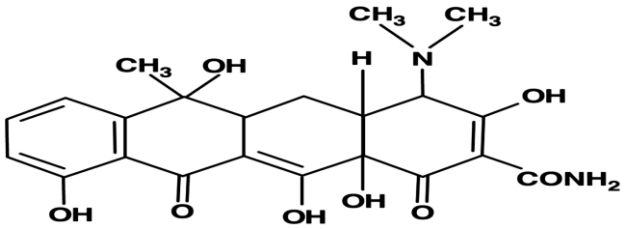


Figure 1.6: Core chemical structure of tetracyclines (Kwiatkowska *et al*., 2013)

They are broad-spectrum antibiotics (Denyer *et al*., 2004) with bacteriostatic activity (Calderon and Sabundayo, 2007; Kwiatkowska *et al*., 2017). They inhibit protein synthesis by reversibly binding to the 30S ribosomal subunit (Calderon and Sabundayo, 2007; Kwiatkowska *et al*., 2017) and by blocking the aminoacyl transfer RNA (tRNA) from entering the acceptor site on the ribosome (Levinson and Jawetz, 2002). Resistance to tetracyclines develops relatively slowly, but there is cross resistance i.e. an organism resistant to one member is usually resistant to all other members of this group (Denyer *et al*., 2004; Calderon and Sabundayo, 2007). Chromosomal mutations in the outer membrane of the organisms that results in decreased penetration of

tetracycline into the cell are a common cause of resistance to tetracyclines (Calderon and Sabundayo, 2007). However, resistance could also be due to an energy-dependent pumping of the drug out of the cell or the biologic/chemical inactivation of tetracyclines by resistant bacteria (Calderon and Sabundayo, 2007).

Most strains of multi-resistant (mr) MRSA (MRSA resistant to two or more classes of antibiotics in addition to β-lactams) in addition to many strains of non-multi-resistant (nmr) MRSA (MRSA susceptible to several or virtually all non-β-lactam antibiotics) are resistant to tetracyclines (Rayner and Munckhof, 2005). However, tetracycline resistant *S. aureus* may still be sensitive to minocycline (Denyer *et al*., 2004). Increase in resistance has led to a decrease in the clinical use of tetracyclines (Denyer *et al*., 2004) and so they are not widely used for the treatment of staphylococcal infections (Rayner and Munckhof, 2005). Candidal superinfection, photosensitivity, gastrointestinal upset as well as discoloration of teeth in children are examples of the adverse side effects of tetracycline use (Rayner and Munckhof, 2005).

1.9.4. Aminoglycosides: Aminoglycosides are naturally occurring antibiotics produced from soil dwelling bacteria (Cox *et al*., 2015). The first identified member of this group of antibiotics is streptomycin (chemical structure as shown in Figure 1.7); however, following its discovery, additional members were identified and semi-synthetic derivatives (e.g. amikacin) developed (Cox *et al*., 2015). Other examples of aminoglycosides include gentamicin, kanamycin, tobramycin and neomycin.

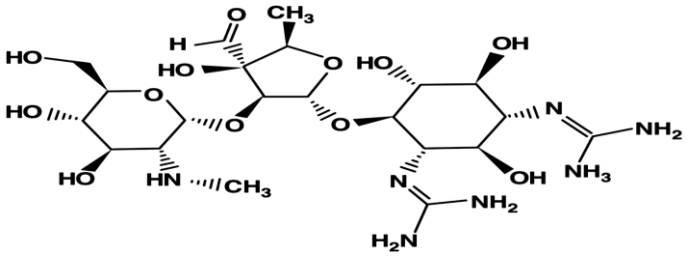


Figure 1.7: Chemical structure of streptomycin (Kwiatkowska *et al*., 2013)

Aminoglycosides are broad spectrum (Etebu and Arikekpar, 2016), bactericidal (Levinson and Jawets, 2002) antibiotics that are able to penetrate the bacterial cell wall and inhibit protein synthesis by irreversibly binding to the 30S ribosomal subunit (Calderon and Sabundayo, 2007; Kwiatkowska *et al*., 2013). Resistance can result from inactivation by enzymes produced by target organism, alterations in the passive diffusion or active transport that hinder the drug from penetrating the bacterial cell wall and chromosomal mutations that make the drug unable to bind to the receptor on the 30S ribosomal subunit (Calderon and Sabundayo, 2007).

Aminoglycosides have limited activity against Gram-positive bacteria and so they are commonly used in combination with a cell wall active agent, e.g. vancomycin or a β-lactam, to treat severe staphylococcal infections (Calderon and Sabundayo, 2007). Many microbiology laboratories use gentamicin susceptibility as a rough guide to likely community acquisition of MRSA because most strains of MSSA and nmrMRSA are susceptible to gentamicin and also because most strains of mrMRSA are gentamicin resistant (Rayner and Munckhof, 2005). Nephrotoxicity and ototoxicity are side effects associated with the use of aminoglycosides (Rayner and Munckhof, 2005; Calderon and Sabundayo, 2007; Kwiatkowska *et al*., 2013).

1.9.5. Oxazolidinones: The P site at the 50S ribosome subunit of bacteria is the molecular target for oxazolidinones; inhibition results in interruption of translation (Vuong *et al*., 2016). Linezolid, a synthetic antimicrobial (chemical structure as shown in Figure 1.8), is the first of the oxazolidinone class (Rayner and Munckhof, 2005).

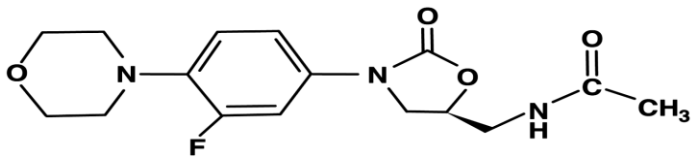


Figure 1.8: Chemical structure of linezolid (Kwiatkowska *et al*., 2013)

Inhibition of bacterial protein synthesis through a mechanism of action different from that of other antimicrobial agents means that cross resistance between linezolid and other classes of antibiotics is not likely (Calderon and Sabundayo, 2007). Linezolid is bacteriostatic against staphylococci (Calderon and Sabundayo, 2007). Resistance to linezolid is rare in/among MRSA, but linezolid is substantially more expensive than conventional therapy (Rayner and Munckhof, 2005). Linezolid is indicated in the treatment of nosocomial pneumonia, skin and soft tissue infection caused by MSSA and MRSA (Calderon and Sabundayo, 2007). It is however reserved for the treatment of patients who are either intolerant of or fail on conventional therapy (Rayner and Munckhof, 2005). Adverse side effects commonly associated with the use of linezolid are headache, diarrhoea, nausea and vomiting (Calderon and Sabundayo, 2007).

1.9.6. Fluoroquinolones: They are fluorinated quinolones (Kwiatkowska *et al*., 2013) that target DNA gyrase and topoisomerase IV (type IIA), which are both essential for bacterial survival (Vuong *et al*., 2016).

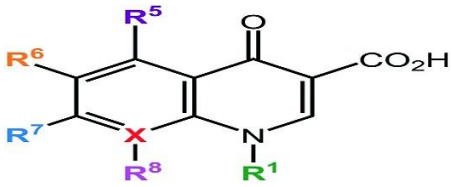


Figure 1.9: Core chemical structure of quinolones; R^1, R^5, R^6, R^7 and R^8 indicate possible sites for structural modification (Pham *et al*., 2019)

Broad-spectrum fluoroquinolones, e.g. ciprofloxacin, moxifloxacin and gatifloxacin, are active against *S. aureus* and exhibit concentration-dependent killing (Rayner and Munckhof, 2005). Rapid emergence of resistance however limits the use of fluoroquinolones as first-line agents in the treatment of staphylococcal infections (Rayner and Munckhof, 2005).

1.9.7. Lincosamides: Are alkyl derivatives of proline and are devoid of a lactone ring (Schmitz *et al*., 1999) (as shown in Figure 1.10).

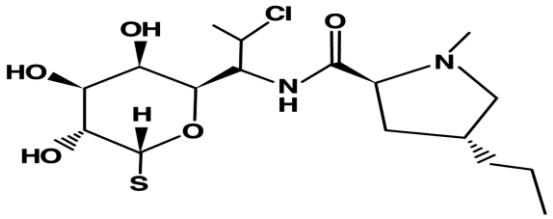


Figure 1.10: Core chemical structure of lincosamides (Kwiatkowska *et al*., 2013)

They inhibit protein synthesis by binding to the 23S rRNA of the 50S subunit (Morar *et al*., 2009). Clindamycin, the most clinically relevant lincosamide (Morar *et al*., 2009) is active against MSSA and most strains of nmrMRSA (Rayner and Munckhof, 2005). It is essentially bacteriostatic and has modest *in vitro* post-antibiotic effect against *S. aureus* (Rayner and Munckhof, 2005). Rapid development of resistance and gastrointestinal side effects have limited the use of clindamycin in the past (Morar *et al*., 2009). Similar to macrolides and streptogramins, alterations in the 23S ribosomal RNA brings about resistance to lincosamides (Tenson *et al*., 2003). However, antibiotic modification is also a common route of resistance to lincosamides (Morar *et al*., 2009).

1.9.8. Streptogramins: Streptogramins inhibit protein synthesis by interfering with peptide bond formation and by blocking the peptide exit tunnel in the 50S ribosomal subunit, thereby preventing the extension of the polypeptide chain (Noeske *et al*., 2014). They are depsipeptides (peptides containing one or more ester bonds within the ring) that consist of chemically distinct types i.e. a smaller type A and a larger type B (Noeske *et al*., 2014) with synergistic inhibitory and bactericidal activity (Schmitz *et al*., 1999). The group/type A streptogramins are responsible for the prevention of peptide bond formation during the chain elongation step while the group/type B streptogramins cause the release of incomplete peptide chains from the 50S ribosomal subunit. Quinupristin/Dalfopristin, a parenteral antimicrobial preparation comprising of two

streptogramins, exerts a time dependent, bacteriostatic (or slow bactericidal) effect and a prolonged *in vitro* post-antibiotic effect against *S. aureus* (Rayner and Munckhof, 2005).

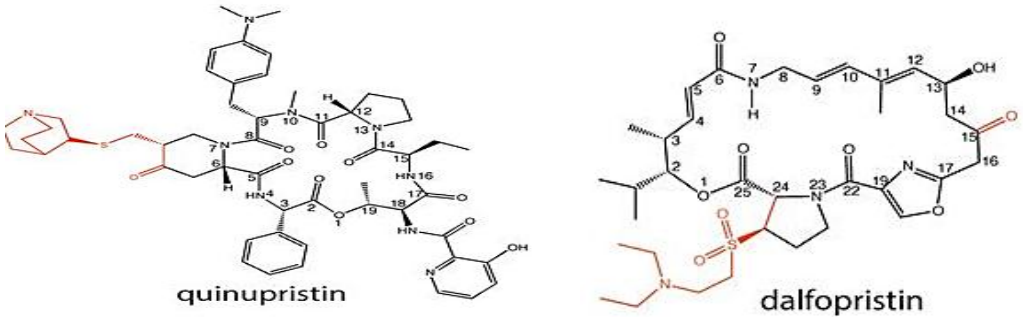


Figure 1.11: Chemical structures of quinupristin (streptogramin B) and dalfopristin (streptogramin A). The coloured side groups are quinuclidinylthiomethyl (quinupristin) and diethylaminoethylsulfonyl (dalfopristin) (modified from Noeske *et al*., 2014)

Quinupristin/Dalfopristin has been indicated for the treatment of suspected or proven MRSA infections requiring intravenous therapy where other antibiotics are inappropriate (Rayner and Munckhof, 2005; Schmitz *et al*., 1999). As with macrolides and lincosamides, alterations in the 23S ribosomal RNA brings about resistance to streptogramins (Tenson *et al*., 2003). Quinupristin/Dalfopristin is very expensive compared with older agents (Rayner and Munckhof, 2005) and is also associated with venous irritation (Schmitz *et al*., 1999). The chemical structures of quinupristin and dalfopristin are as seen in Figure 1.11.

1.10. Methicillin-resistance in *S. aureus*

Methicillin, a semisynthetic antibiotic resistant to β-lactamase inactivation derived from penicillin, was developed to overcome the problem with penicillin-resistant *S. aureus* (Otto, 2012). Infections caused by penicillin-resistant *S. aureus* sharply declined soon after the introduction of methicillin into clinical use (Jevons and Parker, 1964). However, about a year after methicillin's introduction to clinical use by Beecham in 1959, methicillin-resistant *S. aureus* was detected in the United Kingdom (Jevons *et al*., 1963; Chatterjee and Otto, 2013). Thereafter, MRSA has spread and

become endemic in most hospitals worldwide (Lowy, 1998; DeLeo and Chambers, 2009). It has also become established outside the hospital environment, particularly among patients in chronic care facilities and in parenteral drug abusers (Chambers, 1997).

1.10.1. Mechanism of methicillin resistance in *S. aureus*

MRSA are resistant to methicillin and almost all β-lactam antibiotics (Ito *et al.*, 2003; Monecke *et al*., 2016) with the β-lactam antibiotics ceftobipirole and ceftaroline being notable exceptions (Monecke *et al.*, 2016). As with other staphylococci, methicillin resistance in *S. aureus* is caused by the modified penicillin binding protein 2a (PBP2a), which is encoded by allelles of the *mecA* gene (Monecke *et al.*, 2016). The *mecA* gene is responsible for methicillin resistance in staphylococci. *mecA* encodes the 78-kDa penicillin-binding protein 2a (PBP2a) which has low-affinity for methicillin and other β-lactam antibiotics (Utsui and Yokota, 1985; Song *et al*., 1987; Otto, 2012; Otto, 2012b). Penicillin binding proteins are membrane bound enzymes that catalyse the transpeptidation that is necessary for the cross-linkage of peptidoglycan chains (Utsui and Yokota, 1985; Peacock and Paterson, 2015). Unlike other PBPs, PBP2a's active site blocks binding of β-lactams and allows the transpeptidation reaction to proceed (Lim and Strynadka, 2002). Resistance comes about due to the fact that PBP2a substitutes for other PBPs, and because β-lactams cannot inhibit PBP2a, staphylococci are able to survive exposure to high concentrations of these agents (Lowy, 2003; Otto, 2012). Increase in the production of the other PBPs, decrease in the penicillin-binding affinities of the PBPs, or a combination of both accounts for some cases of resistance to β-lactam antibiotics (Hiramatsu, 1995).

The emergence of methicillin-resistant staphylococcal lineages is due to the acquisition and insertion of the SCC*mec* (staphylococcal cassette chromosome *mec*) element into the chromosome of susceptible strains (IWG-SCC, 2009). Staphylococcal cassette chromosome *mec*, is a genomic island of variable size (range 21 to 67 kb) (Hiramatsu *et al*., 2001; Ruppe *et al*., 2009) which

essentially carries the cassette chromosome recombinase (*ccr*) gene complex in addition to the *mec* gene complex (Katayama *et al*., 2000; Zong *et al*., 2011) (as depicted in Figure 1.12).

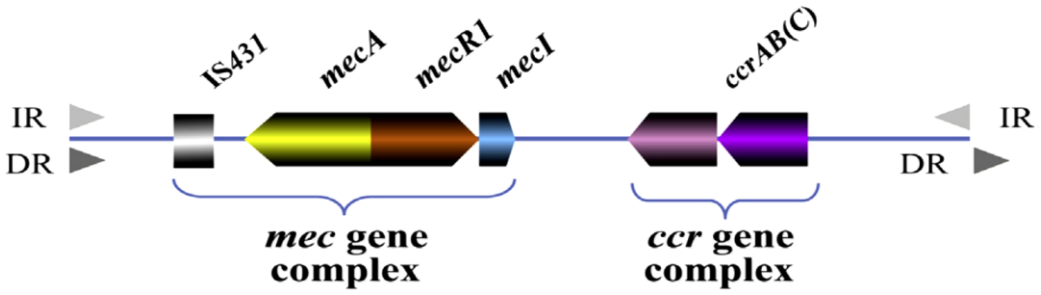


Figure 1.12: Structure of the SCC*mec* element. SCC*mec* bears the mec gene complex (encoding methicillin resistance-*mecA* gene and its regulators *mecI* and *mecR1*), and *ccr* gene complex (encodes the movement, i.e. integration to and precise excision form the chromosome, of the entire SCC*mec* element.
IR: Inverted Repeat; **DR**: Direct Repeat (Hiramatsu *et al*., 2014).

The *mec* gene, its regulatory genes i.e. the *mecR1* sensor inducer and the *mecI* repressor (Shore and Coleman, 2013), and associated insertion sequences (IWG-SCC, 2009) are components of the *mec* gene complex. Ten *mec* complex subclasses, divided into six main classes (A, B, C1, C2, D and E), have been described so far based on the polymorphism of *mecI* and *mecR1* (Kobayushi *et al*., 2001; Zong *et al*., 2011). The *ccr* genes (*ccrC* or the pair of *ccrA* and *ccrB)* encode recombinases responsible for both site- and orientation-specific chromosomal integration and excision of SCC*mec* (Katayama *et al*., 2000; Hanssen and Sollid, 2006; IWG-SCC, 2009). These *ccr* genes and surrounding genes form the *ccr* gene complex (Chen *et al*., 2017). To date, nine *ccr* gene complex types have been described.

In MRSA strains, SCC*mec* elements always integrate sequence specifically at the $attB_{scc}$ (bacterial chromosomal attachment site) located near the origin of replication, at the 3' end of the *orfX* gene (Ito *et al*., 1999; Ito *et al*., 2001; Hiramatsu *et al*., 2002; Ruppe *et al*., 2009). SCC*mec* elements are highly diverse in their structural organisation and genetic content and are classified by a hierarchical system into types and subtypes (IWG-SCC, 2009). Types are defined by the

combination of the type of *ccr* gene complex, and the class of the *mec* gene complex (Ito *et al*., 2001; Ito *et al*., 2004; Chongtrakool *et al*., 2006; IWG-SCC, 2009). Based on the classes of the *mec* gene complex and the *ccr* gene types, 13 types (I to XIII) of SCC*mec* have been assigned for *S. aureus* to date (Baig *et al*., 2018). Types I, II III and VIII are often found in healthcare-associated MRSA (HA-MRSA) strains and they carry additional drug-resistance gene while types IV, V, VI and VII are often found in community-acquired MRSA (CA-MRSA) strains and do not carry any additional drug resistance gene (IWG-SCC, 2009; Kuo *et al*., 2012; Namvar *et al*., 2015). Although SCC*mec* types IX, X and XI have been found in MRSA strains in humans, they are considered to be of animal origin (Shore *et al*., 2012).

Besides the *mec* and *ccr* gene complexes, the SCC*mec* element also contains three so-called J-regions (J1-J3), which constitute non-essential components of the cassette (Hiramatsu *et al*., 2002; IWG-SCC, 2009). Although considered less important in terms of SCC*mec* functions, these regions are epidemiologically significant since they may serve as targets for plasmids or transposons, carrying additional antibiotic and heavy metal resistance determinants (IWG-SCC, 2009; Turlej *et al*., 2011). Variations in the J regions within the same *mec-ccr* complex are used for defining SCC*mec* subtypes (Ma *et al*., 2006; Milheirico *et al*., 2007; IWG-SCC, 2009). While SCC*mec* is crucial for antibiotic resistance, there is no direct evidence that SCC*mec* plays a clear role in MRSA virulence (Stryjewski and Corey, 2014).

Figure 1.13 is a diagrammatic depiction of how methicillin resistance is achieved in *S. aureus* while structures of 13 types of SCC*mec* elements are shown in Figure 1.14.

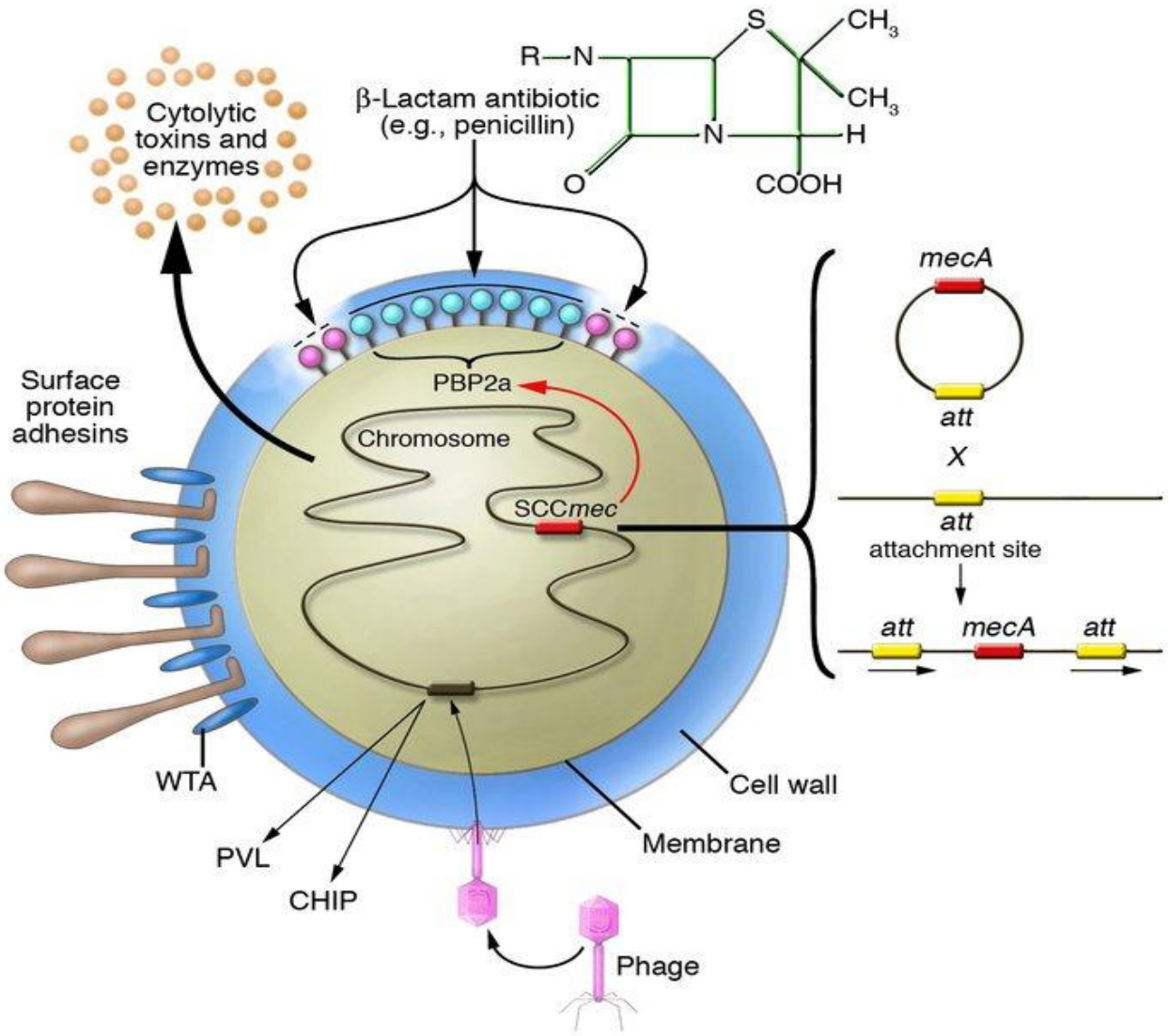


Figure 1.13: Schematic diagram illustrating how *S. aureus* acquires resistance to methicillin and its ability to express different virulence factors. *Staphylococcus aureus* expresses surface protein adhesins and WTA (wall teichoic acid) and also secretes many toxins and enzymes by activation of chromosomal genes. Adhesins and WTA have been implicated in nasal and skin colonization. Resistance to methicillin is acquired by insertion of a horizontally transferred DNA element called SCC*mec*. Five different SCCmec elements can integrate at the same site in the chromosome by a Campbell-type mechanism involving site-specific recombination. The *mecA* gene encodes a novel β-lactam–insensitive penicillin binding protein, PBP2a, which continues to synthesize new cell wall peptidoglycan even when the normal penicillin binding proteins are inhibited. Some virulence factors such as PVL and the chemotaxis inhibitory protein, CHIP, are encoded by genes located on lysogenic bacteriophages (Foster, 2004).

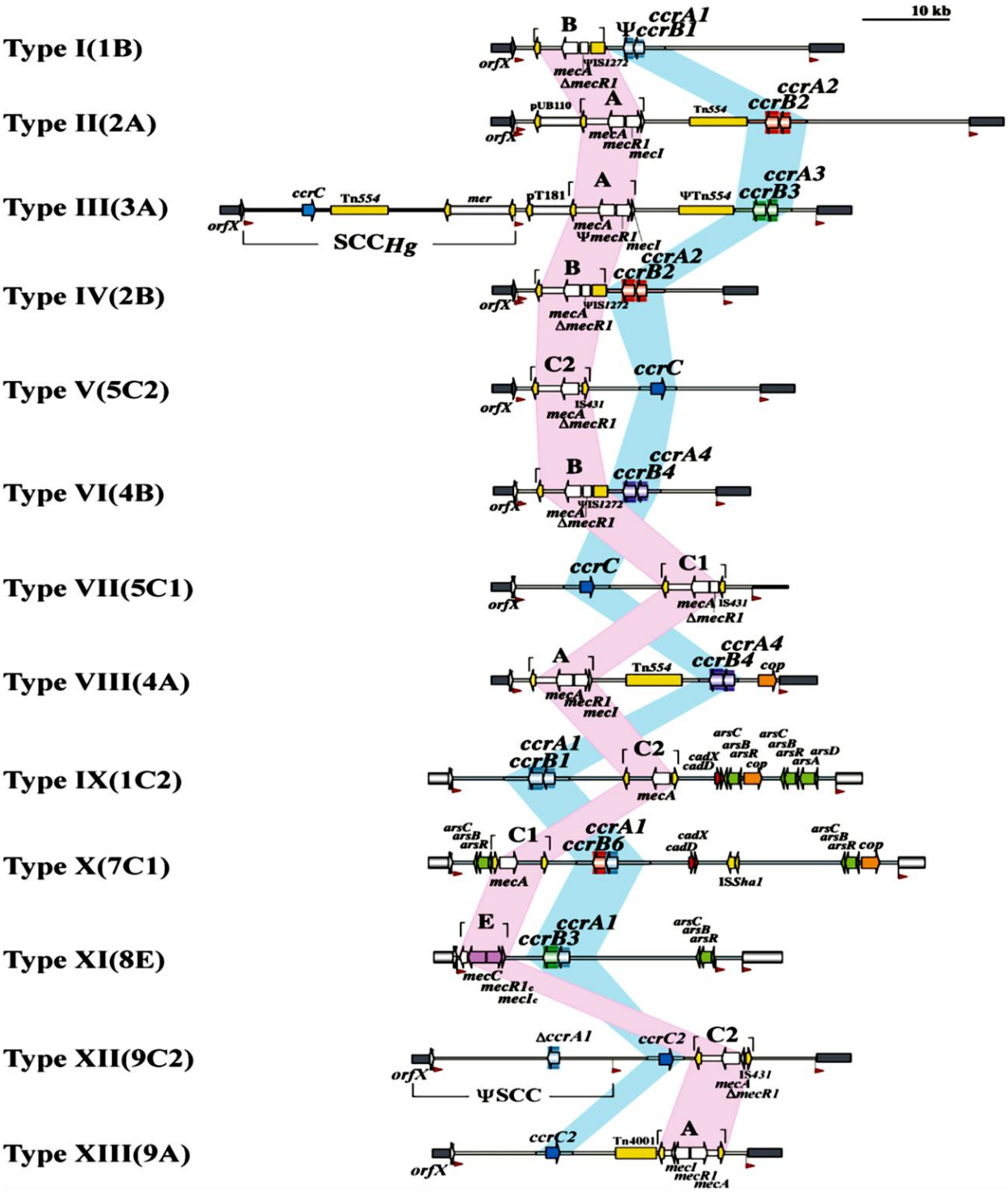


Figure 1.14: Genetic features of SCC*mec* elements I-XIII. Direct repeats that comprise integration site sequences of SCC are located at both extremities of SCCmec (the red arrowheads). The locations of *mec* and *ccr* gene complexes are indicated by the pink and blue belts respectively. Transposons and insertion sequences are indicated in yellow. Representative genes related to heavy metal resistance and integrated plasmids located in the J regions are also indicated (Baig *et al*., 2018).

Early studies, based on structural and nucleotide sequence identity, suggested that the *mecA* gene originated from recombination between a PBP from *E. coli* (Song *et al*., 1987) or from *Enterococcus hirae* (Archer and Niemeyer, 1994) with a β-lactamase encoding gene (Miragaia, 2018). This theory was supported by the finding (by whole genome sequencing) of another *mec* allotype (*mecC*) as part of a class E *mec* complex, containing *blaZ* (*mecI-mecR1-mecC-blaZ*) in *Macrococcus caseolyticus* (Tsubakishita *et al*., 2010A). Other theories have also proposed that the probable evolutionary precursor for the gene encoding PBP2a of MRSA might have originated from *S. sciuri* (Couto *et al*., 1996; Wu *et al*., 2001; Fuda *et al*., 2007). However, more recent data from the work by Tsubakishita *et al*., 2010B, suggest that *S. fleurettii* may be the evolutionary origin of *mecA* in MRSA (Peacock and Paterson, 2015; Lakhundi and Zhang 2018). Both *S. sciuri* and *S. fleuretti* are abundant animal-associated staphylococci (Peacock and Paterson, 2015).

Tsubakishita *et al*., 2010B found that the *mecA* gene of *S. fleurettii* is located on the chromosome linked with the essential genes for the growth of staphylococci and was not associated with SCC*mec*. They also discovered that the *mecA* locus of the *S. fleurettii* chromosome has a sequence practically identical to that of the *mecA*-containing region (~12 kbp long) of SCC*mec*. Thus Tsubakishita *et al*., 2010B speculated that SCC*mec* originated from the *S. fleurettii mecA* gene and its surrounding chromosomal region. Unlike them, Wu *et al*., 2001, found a close homologue of the *mecA* gene of *S. aureus* in *S. sciuri*, however, this homologue did not confer any β-lactam resistance in *S. sciuri*. In an attempt to activate the apparently silent *mecA* gene of *S. sciuri*, they obtained a methicillin resistant derivative through stepwise exposure of the susceptible *S. sciuri* parental strain to increasing concentrations of methicillin. This exposure resulted in a point mutation in the -10 consensus sequence of the promoter, replacing a thymine residue at nucleotide position 1577 in the susceptible *S. sciuri* strain with adenine in the resistant mutant. This resulted in a drastic increase in transcription of the protein that reacted with monoclonal antibodies prepared against the gene product of *mecA* from MRSA, i.e., PBP2a. Transduction of this mutated

mecA into MSSA conferred resistance to the susceptible strains and led to the production of a protein that reacted with the monoclonal antibodies against PBP2a. These observations led Wu *et al*., 2001 to conclude that the *mecA* gene of *S. sciuri* is the probable evolutionary precursor of the *mecA* gene in pathogenic strains of MRSA. Researchers like Fuda *et al*., 2007 also support the argument of the possible acquisition of the pathogenic *mecA* gene from the animal commensal species *S. sciuri*. They compared the biochemical characteristics of the two proteins (i.e. the *mecA* gene product of *S. sciuri* and PBP2a of MRSA) and found them to be highly similar. They found that both proteins were not only similar sequence-wise but also in biochemical behaviour in inhibition by β-lactam antibiotics, the sheltered active site, the need for conformational change in making the active site accessible to the substrate and the inhibitors, as well as the existence of an allosteric site for the binding of bacterial peptidoglycan. These observations, which strongly support the connection between the two proteins, led them to argue in favour of the possible acquisition of the pathogenic *mecA* gene from S. *sciuri*.

Besides *mecA*, other *mec* genes have been identified that are associated with β-lactam resistance, namely (i) *mecC* in *S. aureus*, *Staphylococcus stepanovicii*, *Staphylococcus xylosus*, and *S. sciuri* (Garcia-Alvarez *et al*., 2011; Shore and Coleman, 2013; Loncaric *et al*., 2013; Harrison *et al*., 2013; Harrison *et al*., 2014; Monecke *et al*., 2016); (ii) *mecB* and *mecD* in *Macrococcus caseolyticus* (Baba *et al*., 2009; Miragaia, 2018; Lakhundi and Zhang, 2018). Whereas *mecC* has 69% nucleotide sequence identity with *S. aureus mecA*, the *mecB* and *mecD* respectively have nucleotide identities with *mecA* that are equal or lower than 62% (Miragaia, 2018). All *mec* forms confer resistance to β-lactams to their natural hosts and their introduction into a susceptible *S. aureus* genetic background was able to provide a resistance phenotype, confirming that they should encode a PBP with low-affinity to β-lactams (Baba *et al*., 2009; Kim *et al*., 2012; Miragaia, 2018). Both *mecB* and *mecC* were carried within mobile genetic elements structurally similar to SCC*mec* that were inserted in the *orfX* region (SCC*mec* XI, SCC*mec* IX-like) (Gomez-Sanz *et al*., 2015)

and *mecB* was additionally found within a plasmid in *M. caseolyticus* (Baba *et al*., 2009; Tsubakishita *et al*., 2010A). The *mecD* gene is carried within genomic resistance islands called $McRI_{mecD}$-1, $McRI_{mecD}$-2 (Miragaia, 2018; Lakhundi and Zhang 2018) that are associated with a putative virulence gene and a site-specific integrase, suggesting potential for propagation (Lakhundi and Zhang, 2018). Though the exact evolutionary link between *mecA, mecB, mecC* and *mecD* forms is still undetermined, *mecA* is apparently, the most successful in *Staphylococcus* among all *mec* genes (Miragaia, 2018).

SCC elements that do not carry *mecA* but contain other characteristic genes, such as capsule gene cluster, fusidic acid resistance, or the mercury resistance operon, have also been identified in staphylococci (IWG-SCC, 2009; Lakhundi and Zhang; 2018). However, like SCC*mec*, they carry *ccr* genes in a *ccr* gene complex, they integrate at ISS (integration site sequences) in the staphylococcal chromosome, and they also have flanking DR (direct repeat) sequences containing the ISS (IWG-SCC, 2009; Lakhundi and Zhang; 2018). Furthermore, SCC-like regions similar to SCC that are integrated at ISS and bracketed by ISS but differ from SCC in that they do not harbour a *ccr* gene(s) have also been identified in staphylococci (IWG-SCC, 2009). These regions of varying size (i.e. ranging from 0.1 kb to 34 kb) previously described as SCC-like elements, an arginine catabolic mobile element, a cassette chromosome, or an SCC*mec* insertion site genomic sequence (IWG-SCC, 2009) should be regarded as pseudo-SCC (ΨSCC) elements (IWG-SCC, 2009; Lakhundi and Zhang; 2018). In addition, SCC elements carrying two or more *ccr* gene complexes have been also been identified in various *S. aureus* strains; they are regarded as composite SCC elements (IWG-SCC, 2009; Lakhundi and Zhang; 2018). For example, the SCC*mec* carried by *S. aureus* strain ZH47 is composed of an SCC with *ccrC* and an SCC*mec* carrying class B2 subclass of *mec* gene complex (a subclass of class B *mec* gene complex into which a transposon Tn4001 was integrated), a type 2 *ccr* gene complex and a J1 region with homology to type IVc SCC*mec* (IWG-SCC, 2009; Lakhundi and Zhang; 2018).

1.10.2. Epidemiology of MRSA infections

MRSA is a substantial public health problem worldwide, causing significant morbidity and mortality as well as elevated healthcare costs; MRSA infections are associated with longer hospital stays and an increased financial burden on society (Chu *et al*., 2005; Boucher and Corey, 2008; Thwaites 2010; Filice *et al*., 2010; Green *et al*., 2012). Aside the fear and public distrust about healthcare resulting from the extensive nature of MRSA infections, many healthcare consumers view MRSA bloodstream infection rates as an indicator of quality as well as outcome of care (Kock *et al*., 2010). Due to the fact that only few therapeutic options are available for MRSA infections (Chatterjee and Otto, 2013), the severity of MRSA infections is thought to be higher than that of infections caused by MSSA (Boucher *et al*., 2009; Hanberger *et al*., 2011). Other arguments have also been raised regarding the variation in mortality of MRSA and MSSA infections. It has been suggested that because a large number of older patients with severe underlying diseases contract infections due to MRSA, patient differences account for this variation in mortality (Cosgrove *et al*., 2003; Fowler *et al*., 2005). It has also been suggested that factors such as the virulence of the MRSA organism itself contributes to the difference (Cosgrove *et al*., 2003; Fowler *et al*., 2005).

MRSA was once considered primarily a healthcare-associated threat (Chatterjee and Otto, 2013). However, a dramatic shift in the MRSA target population has occurred as otherwise healthy individuals in the community also develop MRSA infections (DeLeo and Chambers, 2009; DeLeo *et al*., 2010). Distinguishing between healthcare associated (HA) and community associated (CA) MRSA has been problematic (Henderson and Nimmo, 2018). CA-MRSA were traditionally regarded as MRSA that cause infection in previously healthy young patients without prior healthcare contact, susceptible to most non-β-lactam antimicrobial agents, and carrying PVL genes and SCC*mec* types IV or V (Vandenesch *et al*., 2003; Naimi *et al*., 2003; Chambers and Deleo, 2009). Traditional definitions have, however, broken down due to the evolution of the microbiology and epidemiology of CA-MRSA (Otter and French, 2012). For instance, it was once

thought that the presence of some epidemiological markers such as PVL or SCC*mec* type IV in the variable genome could be used to distinguish CA clones (Vandenesch *et al*., 2003). However, the clonal diversity of CA-MRSA now far exceeds that of HA-MRSA with the uptake of diverse SCC*mec* elements into a great variety of genetic backgrounds (Henderson and Nimmo, 2018). Numerous PVL-negative CA-MRSA clones have thus been described and SCC*mec* type IV is no longer exclusive to CA-MRSA and is not present is all clones (Deurenberg and Stobberingh, 2009; Monecke *et al*., 2011).

Definitions based on epidemiological features have commonly been used to distinguish between HA- and CA MRSA (Henderson and Nimmo, 2018). The widely adopted epidemiological definition of CA-MRSA proposed by the US Centres for Disease Control and Prevention (CDC) includes cases where MRSA is isolated less than 48 hrs after hospital admission and there is no history in the previous year of hospitalisation or surgery, permanent indwelling catheters or percutaneous medical devices, residence in a long-term care facility, dialysis or prior culture of MRSA (Morrison *et al*., 2006; Otter and French, 2012; Henderson and Nimmo, 2018). Inclusion of an assessment of previous healthcare contact in the CDC definition means that MRSA linked to a hospitalisation but presenting in the community or at hospital re-admission are classified correctly as HA-MRSA (Otter and French, 2012). HA-MRSA cases are defined as those not meeting the CA-MRSA definition (Henderson and Nimmo, 2018). However, due to the fact that recurrences of CA-MRSA colonisation and infection are common, there will be some misclassification of CA-MRSA as HA-MRSA by epidemiological definitions (David *et al*., 2008; Otter and French 2012; Cluzet *et al*., 2015). Another drawback of epidemiological definitions is the emergence of CA-MRSA clones as an increasingly common cause of HA-infections (Popovich *et al*., 2009; Otter and French, 2012). HA infections, due to clones usually associated with CA infection, have been shown to occur earlier in the course of hospitalisation compared to those due to long established HA-MRSA clones (Maree *et al*., 2007; Henderson and Nimmo, 2018). This

suggests that at least a proportion of infections classified as HA, but due to the more recently emerged CA clones, are endogenous rather than due to spread in hospital (Henderson and Nimmo, 2018).

Millar *et al*., 2007 improved on the CDC definition of CA-MRSA by proposing guidelines for developing a definition of CA-MRSA combining epidemiological factors, antimicrobial susceptibility pattern, clinical presentation and SCC*mec* type. These guidelines however have some shortcomings;

(i) Recommending the use of SCC*mec* typing alone for inferring the MRSA genetic background is not useful for determining the MRSA lineage. This is because isolates with a non-typeable SCC*mec* cassette may be missed and SCC*mec* type IV-carrying HA-MRSA lineages such as ST22-IV (EMRSA-15) and ST5-IV (paediatric clone) may be misclassified as CA-MRSA (Otter *et al*. 2007; Chambers and Deleo, 2009)

(ii) Some CA-MRSA acquired in healthcare settings only meet some of the criteria proposed, in the guidelines, for CA-MRSA (Millar *et al*., 2007).

In 2012, Otter and French proposed that genotyping be included as part of the epidemiological evaluation of CA- and HA-MRSA (Henderson and Nimmo, 2018). However, the introduction of genotypic methods to the definition of MRSA adds extra cost, time and requirement for laboratory equipment, expertise and experience to define these strains (Otter and French, 2012). In practice, characterisation of MRSA clones now requires comprehensive genomic techniques such as micro-array analysis or whole genome sequencing (Henderson and Nimmo, 2018). Nonetheless, ascertaining details of acquisition and onset are necessary for a full understanding of the epidemiology of MRSA (Otter and French, 2012).

CA-MRSA usually cause skin and soft tissue infections (SSTIs) in otherwise healthy individuals (Watkins *et al*., 2012; Kong *et al*., 2016). More severe manifestations can include necrotising

fasciitis (Changchien *et al*., 2011; Kong *et al*., 2016), necrotising pneumonia (Kreienbuehl *et al*., 2011; Kong *et al*., 2016), osteomyelitis (Kechrid *et al*., 2011) sepsis (Bassetti *et al*., 2011), and pyomyositis (Burdette *et al*., 2012). HA-MRSA have most commonly been associated with pneumonia, bacteraemia and other invasive infections in patients exposed to health care settings, who often have co-morbid illnesses (Watkins *et al*., 2012; Kong *et al*., 2016).

Apart from affecting different patient populations and causing distinct clinical syndromes (Watkins *et al*., 2012), several other features also distinguish CA-MRSA from HA-MRSA. They include; (a) increased expression of certain virulence determinants (such as phenol soluble modulins PSMs) in CA-MRSA which may contribute to more severe disease (Wang *et al*., 2007); (b) suggested differences in the biofilm matrix of CA-MRSA (in particular the USA300 lineage) compared to other strains (Kiedrowski *et al*., 2011); (c) they tend to be resistant to fewer non-β-lactam antibiotics; and (d) chromosomal elements for methicillin resistance in CA-MRSA i.e. SCC*mec* (types IV and V) are smaller and more mobile than those found in HA-MRSA (David and Daum, 2010; Cameron *et al*., 2011). Larger SCC*mec* elements (I-III) found in HA-MRSA are associated with reduced bacterial fitness as well as decreased toxin production (Collins *et al*., 2010). Though successful transfer of strains from the hospital to the community and vice versa has occurred, leading to "community-acquired hospital onset" and "hospital-acquired community onset" MRSA infections (Scanvic *et al*., 2001; Klevens *et al*., 2006; Becker *et al*., 2014), traditional hospital-associated strains such as USA100 and USA200 lineages are still highly prevalent in hospitals (Tenover *et al*., 2012). Reports from the US and other parts of the world, however, indicates that HA-MRSA strains are gradually being replaced by CA-MRSA strains in the hospital (Chatterjee and Otto, 2013; Harris *et al*., 2013).

In addition to being a human pathogen, MRSA can also colonise and cause diseases in animals (Chen and Huang, 2018). Cases of livestock associated MRSA (LA-MRSA) infections have been reported as far back as 1972 (Devriese *et al*., 1972; Sharma *et al*., 2016), however, animals were

only considered an important reservoir of MRSA in 2005, when an investigation in the Netherlands revealed a 39% nasal MRSA carriage rate among the Dutch pig populations (Chen and Huang, 2018). Findings from several studies have revealed that two major clones of LA-MRSA, i.e. ST398 and ST9, are in circulation globally (Chen and Huang, 2018). It was found that both clones did not have the human adaption immune-evasion cluster (IEC) genes found in human MRSA strains; thus, this suggested a lower efficiency of the LA-MRSA with regards colonisation and transmission in human beings (Chen and Huang, 2018).

1.10.3. Global epidemic status of MRSA

The most important cause of antibiotic resistant healthcare-associated infections worldwide is MRSA (Chastre *et al*., 2014). It has been estimated that 5% of all hospital-acquired infections are caused by MRSA (Hubner *et al*., 2015). In 1970, it was estimated that MRSA accounted for 2% of *S. aureus* isolates found in hospitals of different countries (O'Toole *et al*., 1970). However, MRSA has become endemic in most hospitals worldwide and accounts for 40-60% of all healthcare-associated *S. aureus* infections (Porto *et al.,* 2013). This intensive spread of MRSA likely occurred due mainly to selective pressure exerted by the high use of antimicrobial drugs in hospitals around the world (Crisostomo *et al*., 2001; Taubes, 2008).

The heavy financial burden of MRSA infections on healthcare systems worldwide can be attributed to prolonged hospital stay and precautionary measures put in place (Hubner *et al.*, 2015). Universal MRSA screening at hospital admission to detect carriers and reduce nosocomial MRSA infection has been advocated (Murthy, 2010). However, universal MRSA screening programmes are costly (Murthy, 2010). It is estimated that MRSA infections within the healthcare setting alone affected more than 170,000 patients annually in the European Union (EU), corresponding to 44% of all infections related to healthcare; these MRSA infections cost EU hospitals an additional 380 million Euros (EUR) annually (Kock *et al*., 2010). Furthermore, estimates put forward that general MRSA infections resulted in 1 million extra days of hospitalisation in the EU, (Gould *et al*., 2010;

Kock *et al*., 2010). In the US, 80,461 cases of invasive MRSA infections with 11,285 related deaths occurred in 2011 and an estimated annual burden of between $1.4 billion and 13.8 billion was attributed to community-acquired MRSA (CDC, 2013; Lee *et al*., 2013). The Asian Network for Surveillance of Resistant Pathogens (ANSORP) study on *S. aureus* infections in Asia showed that MRSA accounted for 25.5% of CA *S. aureus* infections and 67.4% of healthcare-associated (HA) infections (Kim *et al*., 2012). The proportion of MRSA among HA *S. aureus* infections was relatively low in India (22.6%) and the Philippines (38.1%), whereas Sri Lanka (86.5%), Korea (77.6%), and Vietnam (74.1%) showed very high rates of MRSA in the ANSORP study (Kang and Song, 2013).

Despite these, MRSA infection rates are falling in the US and some European countries, including the UK (Stefani *et al*., 2012). For instance, estimates indicate that the number of central line–associated bloodstream infections in US intensive care units (ICUs) decreased from 43,000 in 2001 to 18,000 in 2009, with reductions in infections due to *S. aureus* being more marked than those caused by other pathogens (CDC, 2011). In the UK, implementation of measures such as better antibiotic selection, isolation of infected patients and use of gloves to treat them, including improved hand washing hygiene have helped reduce MRSA infections to a total of 1481 cases reported across the National Health Service between April 2010 and March 2011, representing a 50% reduction from cases reported in 2008 and 2009 (Stryjewski and Corey, 2014).

1.10.3.1. Epidemic status of MRSA in the UK

In the UK, the spread of MRSA infection is higher in intensive care and high dependency units and is also high in neonatal and orthopaedic units (Agha, 2012). The extent of the MRSA epidemic in the UK was monitored by the surveillance of *S. aureus* blood culture-positive cases because they represent the most severe infections and are straight forward to define i.e. blood should be sterile, therefore a positive blood culture likely indicates blood stream infection (Duerden, 2012;

Duerden *et al*., 2015). This surveillance, which involved the reporting of cases by microbiology laboratories to a national database, was initially undertaken by the Public Health Laboratory Service (PHLS) and subsequently its successor, the Health Protection Agency (HPA) between 2003-2013 (Duerden *et al*., 2015). Though initially voluntary, all National Health Service (NHS) hospitals in England were mandated by the government in 2001 to report cases of MRSA blood stream infections (BSIs) (Duerden *et al*., 2015; PHE, 2016). Aside highlighting the burden of MRSA, the information from surveillance is used to monitor and evaluate intervention programs aimed at reducing the burden of MRSA infections (ONS, 2013). In 2011, mandatory surveillance was expanded to also include enhanced surveillance of MSSA data (PHE, 2016).

Multiple infection prevention and control (IPC) initiatives targeting different levels of healthcare were initiated in England to address the challenge of MRSA infection (Duerden *et al*., 2015). The aim of the initiatives was to minimise the risks of infection by the implementation of improved clinical practice protocols (particularly for hand hygiene, environmental cleaning and disinfection) and the implementation of high-impact interventions in the form of care bundles, particularly for invasive procedures with a high risk of infection (Duerden *et al*., 2015). The care bundles or high impact interventions aim to set out in a simple bullet-point format the five or six essential elements needed to minimise the risk of infection associated with invasive procedures (Duerden, 2012). Examples include the Department of Health (DoH) policy document 'Winning Ways', published in 2006 and the 'Saving Lives' programme launched by DoH in 2005 (which included the ambition to halve MRSA rates by 2008) (PHE, 2016). Furthermore, legislation was introduced in 2006, which implemented a statutory Code of Practice on HCAI that applied to all NHS and independent (2008) healthcare providers (Duerden *et al*., 2015). This Code of Practice requires hospitals in England and Wales to have policies for MRSA admission screening and care-pathways for the management of MRSA carriers (Wu *et al*., 2017). Though the identification, isolation and

decontamination of patient carriers is associated with reduced MRSA transmission (Wu *et al*., 2017), MRSA screening represents a significant cost burden for hospitals (Murthy *et al*., 2010). Mandatory surveillance of MRSA BSI in the three years of the target programme (2005-2008) in England showed that there had been a 62% decrease in MRSA BSIs from 7700 in 2003-2004 (baseline) to 2932 in 2008-2009 (Duerden, 2012; Duerden *et al*., 2015). The number further reduced to 1898 in 2009-2010 (75% reduction), 1481 in 2010-2011 (81% reduction) and 1114 in 2011-2012 (86% reduction) (Duerden *et al*., 2015). Acute NHS trusts in England reported a total of 819 cases of MRSA bacteraemia between 2015 - 2016, a 2.4% increase from the 800 cases reported in 2014 – 2015, and an 81.6% decrease from the 4,451 reported cases in 2007 - 2008 (PHE, 2016). The rates of MRSA BSIs per 100,000 population have declined from 8.6 in 2007-2008 to 1.5 in 2015-2016 (as seen in Figure 1.16) (PHE, 2016).

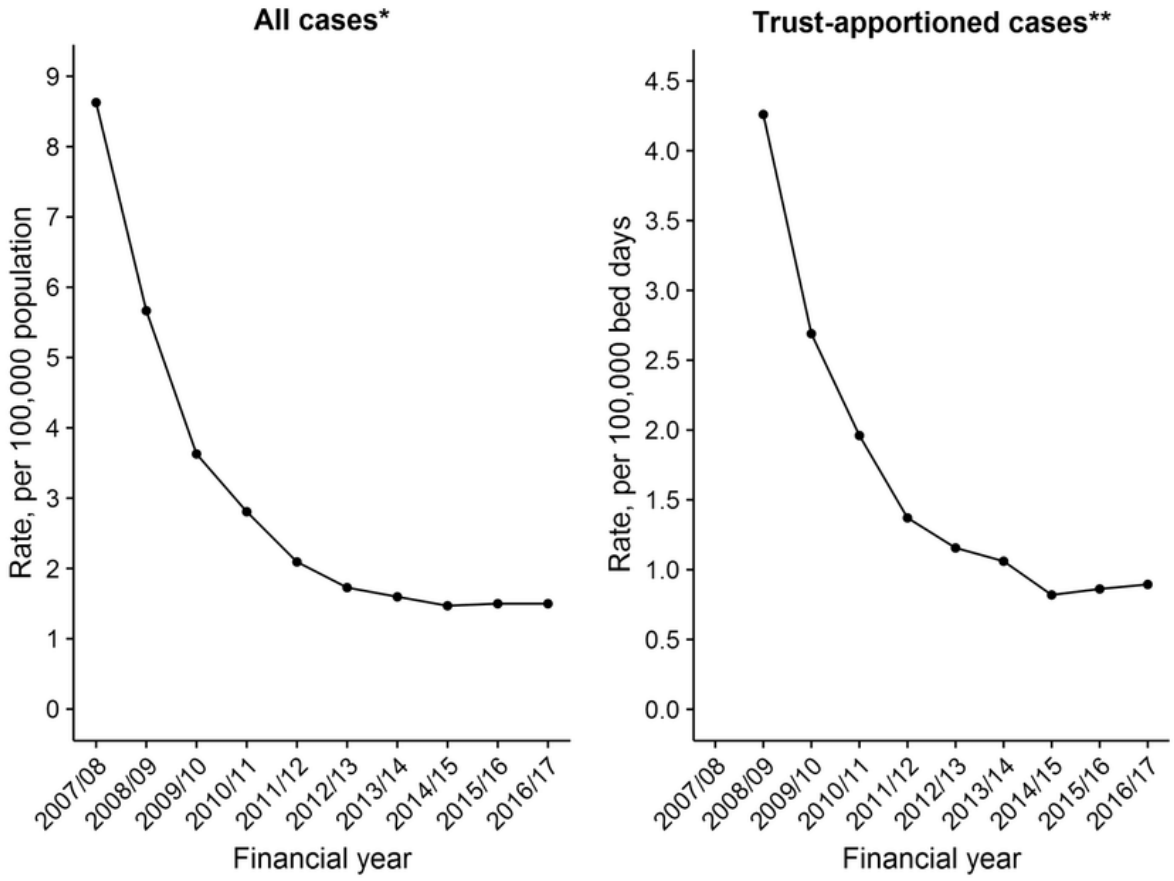


Figure 1.15: Trends in the rate of MRSA bacteraemia in England (PHE, 2016).

* Mid-year population estimates for 2016/17 were not available at time of publication and so population data for 2015/16 was used as a proxy.

** Bed day data was not available for quarter 4 of FY 2016/17 (January to March 2016). As a result, the 2016/17 bed day data is an aggregate of quarters 1, 2 and 3 of 2016/17 and quarter 4 of 2015/16.

$$\left(\text{Rate per 100,000 population} = \frac{n \text{ new cases attributed to CCG in time period}}{\text{Financial year population estimate for time period}} \times 100{,}000\right)$$

$$\left(\text{Rate of CCG assigned MRSA cases, per 100,000 population, FY 2015/16} = \frac{n \text{ CCG assigned cases for CCG, FY 2015/16}}{\text{Financial year population estimate, 2015/16}} \times 100{,}000\right)$$

$$\left(\text{Rate per 100,000 bed days} = \frac{n \text{ cases reported by a Trust, in given time period}}{\text{Average daily occupancy x } n \text{ days in period}} \times 100{,}000\right)$$

There has been a yearly decrease in the number of death certificates in England and Wales mentioning MRSA infection since 2006 when the figure/number peaked at 1,652 (ARHAI, 2014). There was a 20% decrease from 364 in 2011 to 292 in 2012 in the number of death certificates mentioning MRSA while 38 (13%) of the 292 certificates identified MRSA as the underlying cause of death (ONS, 2013).

1.10.3.2. Epidemic status of MRSA in Africa

Though Africa has the highest birth rate and economic growth in some countries compared to the rest of the world, links to global markets, particularly passenger traffic and shipment of goods, are still low (Schaumburg *et al*., 2014). Therefore, interest in the dissemination of emerging pandemic pathogens in Africa might have been low (Schaumburg *et al*., 2014). Data about the epidemiology and prevalence of staphylococcal infections in Africa are scarce compared to information about such infections in the rest of the world (Okon *et al*., 2009). This can be attributed in part to the limited microbiological diagnostic infrastructures in the region (Egyir *et al*., 2015). Several studies have identified *S. aureus* as the main aetiological agent of many infections in sub-Saharan Africa (Olatunji *et al*., 2007; Anguzu *et al*., 2007), yet in Africa and other developing countries, *S. aureus* is still considered to be a less important cause of morbidity and mortality than other infectious diseases such as pneumococcal infections, malaria, human immunodeficiency virus (HIV) infection and tuberculosis (Nickerson *et al*., 2009; Schaumburg *et al*., 2014).

Cases of *S. aureus* related infections (e.g. fatal *S. aureus* pneumonia, complicated skin and soft tissue infection) in travellers returning from Africa were frequently associated with isolates producing PVL (Denis *et al*., 2005; Schleucher *et al*., 2008; Beilouny *et al*., 2008). This suggested that African *S. aureus* might have a different genetic background and might be more virulent than isolates from Europe (Schaumburg *et al*., 2014). Africa is now considered a PVL-endemic region with high rates of PVL-positive isolates, mainly in MSSA ranging from 17% to 74% (Breurec *et*

al., 2011) in contrast to Europe where the prevalence of PVL-positive isolates is low (0.9-1.4%) (von Eiff *et al*., 2004). Prevalence of MRSA in Africa has been shown to vary across different countries ranging from as low as 7% in Madagascar to as high as 82% in Egypt (Omuse *et al*., 2016). This variation in prevalence could be due to different environmental determinants or simply due to difference in the genetic diversity of *S. aureus* (Omuse *et al.*, 2016)

Making definitive relevant conclusions from available evidence regarding the prevalence of methicillin resistance among *S. aureus* isolates in the African countries is difficult because different relevant studies yield variable findings (Falagas *et al*., 2013). Relative limitation in the availability of modern antibiotics effective against HA-MRSA, like linezolid and daptomycin, in most of this part of the world can make the spread of MRSA in the African region worrisome (Falagas *et al*., 2013). Though the spread of pandemic clones of MRSA has major implications for the developing world (Nickerson *et al*., 2009), research into infectious diseases caused by major bacterial pathogens such as *S. aureus* has not yet been sufficiently considered in future research agendas (Schaumburg *et al*., 2014).

1.10.3.3. Epidemic status of MRSA in Nigeria

Since the first detection of MRSA in Nigeria in 1987 (Rotimi *et al*., 1987), there have been reports of MRSA from tertiary health institutions in different regions of the country (Taiwo 2009). Several studies have shown that *S. aureus* is among the most frequently encountered microorganisms in microbiology laboratories in Nigeria (Ako Nai *et al*., 1990; Emele *et al*., 1999; Unachukwu *et al*., 2005). Reports from studies on the prevalence of HA-MRSA in Nigeria have revealed that prevalence varies from one area to another e.g. 43% in Jos, 30.4% in Ibadan, 28.6% in Kano and 12.5% in Maiduguri (Owolabi and Olorioke, 2015). Furthermore, a picture of the prevalence of CA-MRSA in Nigeria is also emerging e.g. 41% in apparently healthy University students in Ekpoma, Edo state, 71.7% in healthy women volunteers in the Abuja Capital Territory and 60.7%

in otherwise healthy inhabitants of Uturu communities in Abia State (Owolabi and Olorioke, 2015). Overall, the prevalence of MRSA infection in Nigeria has been put at less than 50%; available evidence has suggested a 2.3-fold increase in prevalence, i.e. from 18.3% in 2009 to 42.3% in 2013 (Abubakar and Sulaiman, 2018).

No national policy exists for the control, screening and reporting of MRSA outbreaks in Nigeria. Only few institutions carry out limited surveys of MRSA, and because of lack of institutional support such surveys are usually not sustained (Taiwo, 2009). Furthermore, MRSA infections/outbreaks in Nigeria largely go undetected because there is no reference laboratory where MRSA isolated through screening/surveillance can be confirmed genetically and assigned to a clonal group (Taiwo, 2009). Most reports that exist on the characterisation of *S. aureus* in Nigeria were limited to antibiotic susceptibility profiles (Shittu *et al*., 2011) and antibiotic susceptibility testing of *S. aureus* in Nigeria is based on phenotypic testing, especially the disc diffusion technique (Taiwo, 2009; Shittu *et al*., 2011). There is therefore a tendency to underestimate the true prevalence of MRSA from Nigerian studies because standard inoculum disc diffusion tests with oxacillin 1µg disk may not detect some hetero-resistant strains of MRSA (Taiwo, 2009). In addition, only a few studies have investigated the molecular epidemiology of MRSA in Nigeria and this can be attributed to the high cost associated with carrying out molecular studies. As such data on the molecular epidemiology of this pathogen in Nigeria is very limited (Shittu *et al*., 2011).

1.10.4. MRSA clones

Different MRSA clones have emerged ever since the detection of the first MRSA strain in 1959 (Deleo and Chambers, 2009). Majority of MRSA infections are caused by strains belonging to a few clonal complexes (CCs), the most prevalent being CC1 (USA400), CC5 (USA100 or NY/Japan clone, USA800 or pediatric clone), CC8 (Col, Iberian clone, USA300, and USA500),

CC22 (EMRSA-15), CC30 (USA200, EMRSA-16, USA1100, the Southwest Pacific Oceania clone), CC45 (USA600, Berlin clone), CC59 (Taiwan), and CC80 (Europe) (Chatterjee and Otto, 2013).

Most of the epidemic MRSA isolates belong to eight major clonal complexes (CCs) including CC1, CC5, CC8, CC22, CC30, CC45, CC59 and CC80 (Boswihi and Udo, 2018). MRSA strains belonging to CCs 5, 8, 22, 30, and 45, are associated with most HA-MRSA infections (Monecke *et al*., 2011; Stefani *et al.,* 2012; Chatterjee and Otto, 2013; Figueiredo and Ferreira, 2014). Historically, five major internationally disseminated clones have caused HA-MRSA infections. They include Iberian (ST247/SCC*mec*IA), Brazilian (ST239/SCC*mec*IIIA), Hungarian (ST239/SCC*mec*III), NewYork/Japan (ST5/SCC*mec*II) and the Pedriatic (ST5/SCC*mec* IV or IVa) clones (Crisostomo *et al*., 2001; Otto, 2012; Stryjewski and and Corey, 2014). These clones were globally distributed and accounted for the majority (almost 70%) of HA-MRSA infections in several regions (Crisostomo *et al*., 2001; He *et al*., 2010; Stryjewski and Corey, 2014). Other major epidemic MRSA clones such as the archaic clone (ST250/SCC*mec*I), the EMRSA-16 clone (ST36/SCC*mec*II), EMRSA-15 clone (ST22/SCC*mec*IV), and the Berlin clone (ST45/SCC*mec*IVa) have also caused HA-MRSA infections (Yamamoto *et al*., 2012). MRSA strains that belong to CCs 1, 8, 30, and 80, have been associated with CA-MRSA infections (Monecke *et al*., 2011; Chatterjee and Otto, 2013) while those that belong to CCs 97 and 398 have been associated with livestock i.e. LA-MRSA (Boswihi *et al*., 2016).

Some MRSA clones predominate in geographically restricted settings while others have achieved pandemic spread (Monecke *et al*., 2011). For instance, the ST59 and ST93 lineages have limited geographical spread (Boswihi *et al*., 2016) while the CC 8 and 30 MRSA strains are pandemic in both the hospital and community (Chatterjee and Otto, 2013). The ST239/SCC*mec*III MRSA has been widely reported from Asia, Europe, South and North America as well as the Middle East (Monecke *et al*., 2011) while the major clones in the UK are the CC22/SCC*mec*IV (UK-EMRSA)

and CC30/SCC*mec*II (UK EMRSA-16) (Knight *et al*., 2012). Though it's a major clone in the UK, the ST22/SCC*mec* MRSA strain has also been reported in other European countries, Australia, Asia and the Middle East (Boswihi *et al*., 2016). The distribution of MRSA clones in Africa is not well described (Abdulgader *et al*., 2015), however, the predominance of CC8 (ST239 and ST612), CC5 (ST5) and CC30 (ST36) has previously been suggested for Africa (Figuieredo and Ferreira, 2014).

1.10.5. MRSA typing

The changing epidemiology and microbiology of MRSA worldwide (Coia *et al.,* 2014) as well as the high morbidity and mortality associated with MRSA infections have made it necessary to attempt to limit the spread of MRSA by using a range of infection control and prevention interventions (Monecke *et al*., 2011). Thorough knowledge of an organism's epidemiology is the bedrock of effective control measures (Mehndiratta and Bhalla, 2012) and for an epidemiological investigation to be successful there is need for a reliable indicator of the relationship between the organisms isolated i.e. a typing scheme (Weller, 2000). Typing techniques are used to study the population genetics as well as to track sources and pathways of spreading infection (Mehndiratta and Bhalla, 2012). A number of both phenotypic and molecular methods that vary in availability, reproducibility, cost and discriminatory power are available for typing MRSA (Mehndiratta and Bhalla, 2012). However, there is no consensus regarding the best method to use for typing MRSA (Mehndiratta and Bhalla, 2012). Rather, the problem to be solved, the epidemiological context in which the method is going to be used, in addition to the time and geographical scale of use (Sabat *et al*., 2013) dictate the appropriate typing method(s) to be used per time. A typing technique is considered successful when it is inexpensive, highly reproducible, easy to perform and interpret, rapid, with sufficient discriminatory power and widely available (Mehndiratta and Bhalla, 2012; Sabat *et al*., 2013).

Phenotypic typing techniques such as phage typing, serotyping, biotyping, whole cell protein typing and antibiogram typing have been used for many years (Mehndiratta and Bhalla, 2012; Sabat *et al*., 2013). They measure the visible characteristics of tested microorganisms (Kasela and Malm, 2018) and are generally cost effective, widely available, easy to perform and also easy to interpret (Mehndiratta and Bhalla, 2012). The major disadvantage of phenotypic typing techniques is that expression of bacterial genetic material may occur spontaneously or as a response to stimuli from the environment (Kasela and Malm, 2018). Consequently, genetically indistinguishable isolates can have different phenotypes and the unrelated strains may exhibit the same phenotypic properties (Kasela and Malm, 2018). Although, inferior to molecular typing techniques in terms of discriminatory power, reproducibility, typeability and time of analysis, phenotypic typing techniques can still be regarded as irreplaceable tools in the early studies of human pathogens, including *S. aureus* (Kasela and Malm, 2018). Despite their limitations, phenotypic techniques are more available in clinical laboratories and provide an initial identification that allows decision making during an infection (Castro-Escarpulli *et al*., 2015).

Molecular typing techniques assess genome variation in bacterial isolates (Bonofiglio *et al*., 2012) and as such provide more specific information that facilitates epidemiological activities like outbreak investigations, disease surveillance, identifying transmission patterns and risk factors among apparently disparate cases, providing better understanding of disease pathogenesis at the molecular level, prevention and therapy (Foxman and Riley, 2001; Eybpoosh *et al*., 2017). The advantages of nucleic acid-based typing systems lie in the fact that they are less likely to be affected by growth conditions or the laboratory manipulations to which organisms are subjected (Bonofiglio *et al*., 2012). Genetic materials undergo changes due to natural or artificial selective pressures, nevertheless, this mechanism is exactly the basis for their typeability (Bonofiglio *et al*., 2012). A few of the most commonly used molecular typing techniques are described below.

1.10.5.1. Pulsed field gel electrophoresis (PFGE): This is a non-sequence based genotyping method that is based on DNA banding pattern (Fournier *et al*., 2014). It was once considered the 'gold standard' for MRSA typing (Lakhundi and Zhang, 2018) and is based on the generation of large chromosomal fragments by digestion with the low frequency cutting *Serratia marcescens* endonuclease I (*Sma*I) enzyme (Mandell *et al*., 2009; Alkharsah *et al*., 2018). Generated fragments are separated in agarose gel by changing the orientation of the electric field periodically (Mehndiratta and Bhalla, 2012) to yield banding patterns specific for particular clones (Mandell *et al*., 2009). Although PFGE is widely used, has excellent typeability and discriminatory power in addition to high reproducibility at the inter-laboratory level when standardised protocols are used (Rasschaert *et al*., 2009; Mehndiratta and Bhalla, 2012; Sabat *et al*., 2013; Omar *et al*., 2014), it does not produce information on the genealogy of an organism (Mandell *et al*., 2009; Struelens *et al*., 2009), is time-consuming, technically demanding, labour-intensive and may lack the resolution power to distinguish bands of nearly identical size (Sabat *et al*., 2013). PFGE has been found to be extremely useful in the characterisation of outbreak strains (Mehndiratta and Bhalla, 2012; Lakhundi and Zhang, 2018), however, it has been reported that the stability of PFGE is not sufficient for reliable application in long term epidemiological surveillance due to long evolutionary history of pandemic clones (Mehndiratta and Bhalla, 2012). It is important to note that animal-associated MRSA is not typeable by this method as the activity of *Sma*I is blocked due to methylation of the restriction site (Bens *et al*., 2006; Struelens *et al*., 2009; Marek *et al*., 2018).

1.10.5.2. Multilocus sequence typing (MLST): This is a sequence-based method that allows for the unambiguous assignment of the ancestral phylogeny of the staphylococcal population (Enright *et al*., 2002). MLST consists of the sequencing of seven housekeeping genes i.e., *arcC, aroF, glpF, gmk, pta, tpi and yqiY* (Mandell *et al.*, 2009; Mehndiratta and Bhalla, 2012). They encode the enzymes carbamate kinase, shikimate dehydrogenase, glycerol kinase, guanylate kinase, phosphate acetyltransferase, triosephosphate isomerase and acetyl-coenzyme A

acetyltransferase respectively (Vivas, 2018). The different sequences generated are assigned distinct alleles of each housekeeping gene to generate an allelic profile or sequence type (ST) which defines an isolate (Deurenberg *et al*., 2007; Vivas and Gutierrez, 2018). When five of the seven housekeeping genes have identical sequences, *S. aureus* strains are grouped within a single CC (Deurenberg *et al*., 2007; Vivas and Gutierrez, 2018). The housekeeping genes are independent of antibiotic resistance genes and as such MLST traces staphylococci to their antibiotic susceptible ancestors (Mandell *et al*., 2009). MLST is highly reproducible (Sabat *et al*., 2013), but it also has weak discriminatory power, is time consuming, labour intensive and expensive thereby limiting its use for epidemiological studies (Rasschaert *et al*., 2009, Sabat *et al*., 2013).

1.10.5.3. ***Spa* typing**: This method involves the sequencing of the variable number tandem repeat (VNTR) region (i.e. the polymorphic X region) of the *S. aureus spa* gene (Shore *et al*., 2012; Sabat *et al*., 2013). The high degree of genetic diversity in the VNTR region of the *spa* gene results from both a variable number of short repeats (24 bp) and various point mutations (Sabat *et al*., 2013). *Spa* typing has lower discriminatory ability than PFGE (Sabat *et al*., 2013), but provides greater discrimination than MLST (Shore *et al*., 2012). It is currently the most useful instrument for characterising *S. aureus* isolates at the local, national and international levels because of its ease of use, speed, appropriate *in vivo* and *in vitro* stability, excellent reproducibility, cost-effectiveness, standardised international nomenclature, high-throughput by using the StaphType software, and full portability of data via the Ridom database (http://spaserver.ridom.de) (Hallin *et al*., 2007; Grundmann *et al*., 2010; Mehndiratta and Bhalla, 2012; Shore *et al*., 2012). *Spa* typing's major limitation is the misclassification of particular types due to horizontal DNA transfer and recombination and/or homoplasy (Nubel *et al*., 2008; Sabat *et al*., 2013).

1.10.5.4. **SCC*mec* typing:** SCC*mec* typing commonly relies on the use of several multiplex PCR assays to identify the *ccr* and *mec* complex genes which are used to define the SCC*mec* type

and to identify the main characteristics of the J regions for SCC*mec* subtype determination (Shore *et al*., 2012; Mehndiratta and Bhalla, 2012). Some other SCC*mec* typing strategies rely on sequence determination of internal fragments of recombinase genes (Struelens *et al*., 2009; Mehndiratta and Bhalla, 2012) while complete nucleotide sequencing is commonly carried out in the case of novel SCC*mec* elements (Shore *et al*., 2012). Accurate identification of the type and subtype of the SCC*mec* element harboured by an MRSA isolate enhances understanding of the genetic relatedness of MRSA isolates (Shore *et al*., 2012).

1.10.5.5. Whole Genome Sequencing (WGS): WGS of bacteria using next generation sequencing (NGS) is the ultimate discriminatory sequence based genotyping method (Fournier *et al*., 2014). Like other NGS technologies, the WGS technique can generate millions of reads (approximately 35–700 bp in length) in single runs at comparatively low costs (Sabat *et al*., 2013). The generated multiple short sequence reads are then assembled based on overlapping regions (de novo assembly), or comparisons with previously sequenced 'reference' genomes (resequencing) to generate the complete nucleotide sequence of a genome (Sabat *et al*., 2013). WGS has shown superior resolution compared with standard typing techniques such as the PFGE and *spa* typing techniques when used for individual outbreaks (Gordon *et al*., 2017), has sufficient discriminatory power to reconstruct local and intercontinental transmission of MRSA strains (Harris *et al*., 2013) and can also provide additional information about population structure, resistance and pathogenicity (Gordon *et al*., 2017).

1.10.5.6. DNA Microarray profiling: DNA microarray systems allow simultaneous hybridisation of an isolate's genome against the entire content of multiple *S. aureus* genomes (Shore *et al*., 2012). This typing technique involves the use of a collection of DNA probes attached to a solid surface in an ordered fashion (Butte, 2002; Sabat *et al*., 2013). These probes, which may be short oligonucleotides or PCR products (Garaizer *et al*., 2006; Sabat *et al*., 2013), are used to

detect complementary nucleotide sequences in the genomes of bacterial strains (Sabat *et al*., 2013). Microarrays can be used to detect genes that serve as specific markers for particular bacterial strains in addition to detecting the allelic variants of a gene present in all strains of a specific species (Sabat *et al*., 2013). However, analysis of data generated by microarrays is complicated, as such, their use is restricted mainly to specialised research laboratories (Shore *et al*., 2012).

1.10.6. Treatment options for MRSA infections

Staphylococcus aureus has been identified as one of the key 'problem' bacteria in addition to *Enterococcus faecium*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* species (the ESKAPE pathogens) that urgently require development of new therapies (Holmes *et al*., 2015). The ideal antibiotic agent for the treatment of MRSA should have the following properties; excellent tissue penetration, consistent pharmacokinetics and pharmacodynamics that allow for predictable dosing, rapid bactericidal killing, low potential for the development of resistance while on therapy, low side effect profile and demonstrated clinical and microbiological efficacy (Nguyen and Graber, 2010). Such agent does not exist, and combination therapy has been advocated to fill in the gaps where each individual agent fails (Nguyen and Graber, 2010).

Many of the new antimicrobial agents available for the treatment of MRSA infections are associated with dose-limiting adverse events, emerging resistance issues, and high drug costs (Rodvold and McConeghy, 2014). Oral agents such as clindamycin, trimethoprim/sulfamethoxazole (TMP-SMX), doxycycline, and minocycline are used primarily to treat CA-MRSA SSTIs in outpatient settings (Moellering, 2008; Gould *et al*., 2012).

Theoretically, trimethoprim/sulfamethoxazole may be less effective in clinical settings where there is undrained pus, as *S. aureus* may be able to scavenge thymidine from dead inflammatory cells and injured tissues and thus bypass the thymidine biosynthesis pathway ordinarily blocked by

trimethoprim/sulfamethoxazole (Proctor, 2008). The use of TMP-SMX to treat CA-MRSA infections is primarily based on a 1992 study by Markowitz and his colleagues (Markowitz *et al*., 1992) that compared intravenous trimethoprim/sulfamethoxazole with vancomycin in 101 injection drug users with *S. aureus* infections requiring hospitalisation (Markowitz *et al*., 1992). Vancomycin was found to be superior with regard to duration of bacteraemia, sterilisation of wound culture, duration of fever and clinical failure rates but the differences in efficacy were largely seen among patients infected with MSSA (Markowitz *et al*., 1992). Therefore, TMP-SMX may be considered as an alternative to vancomycin in selected cases of MRSA infection (Markowitz *et al*., 1992). The high rates of drug rash and hypersensitivity reactions associated with TMP-SMX may also limit its clinical utility (Rodvold and McConeghy, 2014).

Use of clindamycin is limited among HA-MRSA clones because of its bacteriostatic nature, observed high rate of resistance (both inducible and constitutive) and its ability to predispose to *Closteroides difficile*-associated colitis (Nguyen and Graber, 2010). There exist clones of CA-MRSA that are mostly susceptible to clindamycin thereby allowing for consideration of its use in infections caused by CA-MRSA (Nguyen and Graber, 2010) Clindamycin has been widely used by paediatricians for therapy of skin and soft-tissue infections, including those due to CA-MRSA (Daum, 2007). Use of doxycycline and minocycline in adolescents and adults is limited due to the risks of staining dental enamel in children aged less than 12 years (Rodvold and McConeghy, 2014). They are bacteriostatic against *S. aureus* (Nguyen and Graber, 2010) and have potential photosensitisation potential (Rodvold and McConeghy, 2014). Experience in their use in severe *S. aureus* infections is largely anecdotal (Ruhe *et al*., 2005).

Despite the broad-spectrum resistance to β-lactams conferred by PBP2a, some newer cephalosporin β-lactams, e.g. ceftaroline (formerly referred to as PPI-0903M, T-91825, TAK-599) (Duplessis and Crum-Cianflone, 2011) and ceftobiprole (formerly BAL9141, BAL5788, and RO-

63-9141) (Pfaller *et al*., 2019), are effective against MRSA (Peacock and Paterson, 2015). Both ceftaroline and ceftobiprole have broad-spectrum activity against a range of gram-positive and gram-negative bacteria, including MRSA (Duplessis and Crum-Cianflone, 2011; Peacock and Paterson, 2015; Pfaller *et al*., 2019) and are beginning to be used clinically to combat these infections (Peacock and Paterson, 2015). Both are effective against MRSA in vitro and have significantly lower MIC values compared with those of other β-lactams (Peacock and Paterson, 2015). This can be attributed to the fact that both ceftaroline and ceftobiprole have a significantly higher affinity for PBP2a (Davies *et al*., 2007; Kosowska-Shick *et al*., 2010; Moisan *et al*., 2010). Biochemical and structural studies have shown that ceftaroline is an allosteric activator of PBP2a in that it binds and activates the allosteric site by virtue of its D-Ala–D-Ala mimicry, thereby promoting the active-site binding of a second ceftaroline molecule (Duplessis and Crum-Cianflone, 2011; Villegas-Estrada *et al*., 2008; Otero *et al*., 2013). However, whether ceftobiprole also acts in the same way is unclear (Peacock and Paterson, 2015). Unfortunately, resistance to ceftaroline has been observed at a low frequency among MRSA isolates; this resistance is associated with mutations in the PBP2a transpeptidase domain, which act to lower the binding of ceftaroline (Mendes *et al*., 2012; Alm *et al*., 2014). Mutations in the allosteric domain have also been observed (Villegas-Estrada *et al*., 2008; Mendes *et al*., 2012; Alm *et al*., 2014).

Characteristics of some other parenteral agents available for the treatment of serious MRSA infections are as discussed below.

1.10.6.1. Vancomycin

Unlike other antibiotics, MRSA took almost 40 years to develop even partial resistance to glycopeptides such as vancomycin (Stryjewski and Corey, 2014). There is ongoing debate as to whether vancomycin is obsolete, but, treatment guidelines and surveys indicate vancomycin remains the workhorse of parenteral anti-infective agents for MRSA infections even after more

than 50 years of clinical use (Mohr *et al*., 2007; Yang *et al*., 2010; Gould *et al*., 2012). Vancomycin intermediate-susceptible *S. aureus* (VISA) was first described in Japan in 1997 following which additional cases were subsequently reported from other countries (Hiramatsu *et al*., 1997; Smith *et al*., 1999). The mechanism of resistance in VISA involves a thicker cell wall with an excess of binding sites able to "trap" glycopeptides (Hanaki *et al*., 1998; Hanaki *et al*., 1998b) and is commonly related to previous exposure to vancomycin (Hanaki *et al*., 1998b). Results from a study indicates that VISA strains frequently carry SCC*mec* type II and are usually susceptible (>90%) to linezolid, telavancin, tigecycline, and minocycline (Saravolatz *et al*., 2012). Moreover, VISA isolates can return *in vitro* to vancomycin susceptible when the antibiotic pressure is removed (Boyle-Vavra *et al*., 2000). Full vancomycin-resistant *S. aureus* (VRSA) emerged clinically in 2002 and are extremely uncommon possibly due to increased fitness cost associated with high-level resistance to vancomycin (Otto, 2012). Unlike VISA, the mechanism of resistance in VRSA is due to acquisition of the *vanA* operon transferred from vancomycin-resistant enterococci (Lowy, 2003). Not long after VISA was described, reports arose of a pre-VISA stage of resistance i.e. heterogeneously resistant strains of MRSA, which remain susceptible to vancomycin but contain resistant subpopulations resistant to glycopeptides (Lowy, 2003). They are not detected by conventional laboratory methods (Stryjewski and Corey, 2014).

Most clinicians use vancomycin for the empiric and definitive therapy of systemic MRSA infections (Liu *et al*., 2011). It has become obvious that no single factor can serve as a predictor of success or failure for vancomycin therapy (Kullar *et al*., 2011). Several observations over time have confirmed or suggested that; (i) higher rates of nephrotoxicity (e.g. 20%–30%) are associated with high-dose vancomycin therapy (Wong-Beringer *et al*., 2011); (ii) higher vancomycin minimum inhibitory concentration (MIC) values for isolates of MSSA and MRSA appear to influence clinical outcomes associated with beta-lactam agents and vancomycin, respectively (Holmes *et al*., 2011); (iii) vancomycin MIC values are method specific (Hsu *et al*., 2008) and that;

(iv) clinical outcomes with vancomycin are a function of bacterial load at the site of infection (Walraven *et al*., 2011).

1.10.6.2. Telavancin

Telavancin, a new drug approved for the treatment of adult patients with complicated skin and skin structure infections (SSSIs), including MSSA and MRSA is a once-daily parenteral lipoglycopeptide (Rodvold and McConeghy, 2014). Telavancin activity is due to the novel combined action of disruption of bacterial cell membrane barrier function/permeability and inhibition of cell wall synthesis (Boswihi and Edet, 2018). It was approved by the United States Food and Drug Administration (FDA) in June 2013 for limited use when no other options are available for the indication of nosocomial pneumonia (including ventilator associated pneumonia) caused by Gram-positive bacteria, including MRSA (Figueiredo and Ferreira, 2014; Rodvold and McConeghy, 2014). In light of the following warnings and precautions, careful consideration is required before using telavancin for acute bacterial skin and skin structure infections:

(i) More frequent increases in serum creatinine to 1.5 times baseline values (15% vs 7%) and higher rates of renal impairment (3.1% vs 1.1%) compared to vancomycin;

(ii) Decreased efficacy in patients with reduced renal function compared to vancomycin;

(iii) Lack of data and unknown efficacy in life-threatening bloodstream infections

(Rodvold and McConeghy, 2014).

1.10.6.3. Quinupristin-dalfopristin

Quinupristin/dalfopristin, a streptogramin combination, is bacteriostatic against MRSA that express streptogramin resistance mediated by the macrolide–lincosamide–streptogramin (MLS_B) resistance complex but is bactericidal against MRSA isolates that do not express MLS_B resistance (Low and Nadler, 1997). This phenomenon is relatively common among HA-MRSA isolates

(Schmitz *et al*., 1999) but also is also emerging in CA-MRSA isolates as well (Nguyen and Graber, 2010). Quinupristin-dalfopristin has limited utility in patients with serious MRSA infections because of resistance issues (Saravolatz and Eliopoulos, 2003); resistance of vancomycin-resistant enterococci to quinupristin/dalfopristin emerged fairly rapidly after its clinical introduction (Corti and Imhof, 2009) . Furthermore, *in vitro* antagonism between QD and other antibiotics has been described. This was observed in the 2001 time-kill studies carried out by Fuchs *et al*., on *S. aureus* isolates with constitutive MLS_B resistance. When these isolates were exposed to the combination of QD and eight other antimicrobial agents (i.e. vancomycin, cefepime, ceftazidime, imipenem, piperacillin-tazobactam, and ciprofloxacin), the killing rates were reduced compared to when the eight were tested alone. In addition, increased risk of adverse events, e.g. myalgias, and arthralgias, following treatment with QD have also been described (Drew *et al*., 2000 and Raad *et al*., 2001).

1.10.6.4. Linezolid

Linezolid, a synthetic antibiotic belonging to the oxazolidone class (Nguyen and Graber, 2010), inhibits protein synthesis by binding to the ribosomal RNA (Boswihi and Edet, 2018). It is an oral agent available for outpatient and step-down therapy of MRSA infections (Shaw and Barbachyn, 2011). Linezolid is 100% bioavailable and demonstrates extensive tissue penetration, including into the epithelial lining fluid of the lungs and infected skin and soft tissues of diabetic patients (Liu *et al*., 2011). Published guidelines for the treatment of MRSA consider linezolid an alternative first-line agent for MRSA pneumonia (Liu *et al*., 2011), but it has been generally avoided as front-line treatment for MRSA endocarditis bacteraemia because of its bacteriostatic nature (Nguyen and Graber, 2010). Even though higher cure rates have been found for linezolid compared to vancomycin, whether linezolid is superior to vancomycin for its approved indications (in complicated and uncomplicated SSSI and nosocomial pneumonia) remains controversial (Rodvold and McConeghy, 2014). It is important to mention that MRSA resistance to linezolid has been described in clinical settings albeit uncommon (Stryjewski and Corey, 2014). Outbreaks of

healthcare-associated linezolid-resistant infections have been associated with ribosomal point mutations in the 23S rRNA gene, or ribosomal proteins L3 and L4, in several countries (Gu *et al*., 2013). A plasmid-borne methyltransferase-mediated resistance mechanism cfr (for chloramphenicol-florfenicol resistance gene) has also been shown to convey resistance to linezolid (Gu *et al*., 2013). Drug-related side effects observed with linezolid include diarrhoea, nausea, headache, abnormal liver function, reversible myelosuppression (including thrombocytopenia, leucopenia, pancytopenia, and anaemia) (Rodvold and McConeghy, 2014). Other rare but serious adverse events include lactic acidosis, peripheral neuropathy, and optic neuropathy, mostly with prolonged therapy (>28 days) (Rodvold and McConeghy, 2014). Serotonin syndrome has been reported in cases with concomitant administration of selective serotonin reuptake inhibitors (Palenzuela *et al*., 2005; Rodvold and McConeghy, 2014).

1.10.6.5. Daptomycin

Daptomycin, a cyclic lipopeptide with activity against Gram positive organisms including MRSA and vancomycin-resistant enterococci (Schriever *et al*., 2005), disrupts cell membrane function via calcium-dependent binding, thus resulting in bactericidal activity in a concentration dependent fashion (Choo and Chambers, 2016). Daptomycin is recommended as an intravenous option for complicated SSSIs (Gould *et al*., 2012). It is also recommended as an alternative to vancomycin for patients with MRSA bacteraemia (6 mg/kg once daily) and native-valve, right-sided infective endocarditis (Fowler *et al*., 2006; Gould *et al*., 2012). However, daptomycin is ineffective for pneumonia as it is inactivated by lung surfactant (Silverman *et al*., 2005; Enoch *et al*., 2007). Daptomycin resistance results from mutations in genes that activate the defences of the bacterium against damage to the cell envelope including host cationic antimicrobial peptides (Bayer *et al*., 2013; Miller *et al*., 2016) Cases of daptomycin-resistant staphylococci are generally seen in high-inoculum infections (e.g. infective endocarditis and abscesses) (Tran *et al*., 2015; Foster, 2017) and when lower doses of the drug have been used (i.e. ≤ 6 mg/kg/day) (Tran *et al*., 2015).

Furthermore, several studies have linked the prior use of vancomycin associated with development of the vancomycin-intermediate *S. aureus* (VISA) phenotype with increased resistance to daptomycin during therapy (Patel *et al*., 2006; Fowler *et al*., 2006; Pillai *et al*., 2007; van Hal *et al*., 2011; Bayer *et al*., 2013). It has been recommended that high-dose daptomycin (10 mg/kg once daily) in combination with other antibiotics be administered for persistent MRSA bacteraemia when isolates are susceptible to daptomycin or when organisms have a high vancomycin MIC or for vancomycin-resistant strains (Gould *et al.*, 2012).

1.10.6.6. Tigecycline

Tigecycline is a semisynthetic derivative of minocycline and the first licensed drug from the glycylcycline class of antimicrobial agents (Livermore, 2005; Slover *et al*., 2007) with broad spectrum of activity (Rodvold and McConeghy, 2014; Choo and Chambers, 2016). It is highly protein bound, resulting in low serum levels that limit its effectiveness when bacteraemia is present (Rose *et al*., 2006). However, tigecycline has shown promise in the treatment of complicated SSTIs and intra-abdominal infections caused by MRSA (Babinchak *et al*., 2005; Florescu *et al*., 2008; Wang *et al*., 2017). Despite repeated findings of non-inferiority, the US Food and Drug Administration (FDA) in 2012 issued a warning citing an increased mortality risk associated with tigecycline (FDA, 2012; Prasad *et al*., 2012). Findings from 13 trials of approved and unapproved indications, revealed that tigecycline had an overall adjusted mortality rate of 4% compared with 3% for the comparator group, a relative mortality increase of 33% (FDA, 2012; Prasad *et al*., 2012). Although the FDA emphasised that the risk was most clearly seen in patients with hospital acquired pneumonia, for which tigecycline is not approved, they also acknowledged that excess mortality was also seen in patients treated for approved indications (Prasad *et al*., 2012). The cause of excess deaths could not be determined, nevertheless, it was postulated that most deaths were secondary to progression of infection (FDA, 2012; Prasad *et al*., 2012).

1.10.6.7. Quinolones

Quinolones originated in the 1960s as a by-product from the synthesis of antimalarial quinines and by the 1980s, fluorinated derivatives such as ofloxacin, ciprofloxacin and norfloxacin had appeared (Mandell *et al*., 2009). Fluoroquinolones were initially introduced for the treatment of Gram-negative bacterial infections (Lowy, 2003), but they were also seen to be active against Gram-positive bacteria (Mandell *et al*., 2009). Quinolone resistance among *S. aureus* is more prominent among MRSA strains; as a result, the ability to use fluoroquinolones as anti-staphylococcal agents is dramatically reduced (Lowy, 2003). Resistance to fluoroquinolone develops because of spontaneous chromosomal mutations in the target of the antibiotic, topoisomerase IV or DNA gyrase, or by the induction of a multidrug efflux pump (Lowy, 2003). The most common types of mutational change result in amino acid substitutions in residues that constitute the drug binding site, the so-called quinolone resistance-determining region (Hooper and Jacoby, 2015). ParC is the topoisomerase with the greatest sensitivity, therefore, it is the primary target; on the other hand, DNA gyrase is less susceptible and is thus the secondary target (Hooper and Jacoby, 2015; Foster, 2017). Fluoroquinolone-resistant isolates often overexpress chromosomally encoded efflux pumps (Hooper and Jacoby 2015), i.e. i.e. NorA, NorB and NorC (Foster, 2017).

1.10.7. Potential new treatment option for *S. aureus* infections: Iron-chelators and Iron-chelation therapy

With the exception of organisms such as the Lactobacilli and *Borrelia burgdorferi*, iron is absolutely required for life of all forms as it participates in major biological processes such as gene regulation, DNA synthesis, respiration, oxygen transport, the trichloroacetic acid (TCA) cycle, etc. (Andrews *et al*., 2003). *In vivo*, mammals maintain low levels of circulating free iron by means of iron-binding proteins such as the extracellular lactoferrin and transferrin as well as the intracellular ferritin, haeme and haemosiderin (Sritharan, 2006; Cassat and Skaar, 2013). In addition to

mitigating toxicity associated with excess iron (Cassat and Skaar, 2013), low iron-availability provides a non-specific defense mechanism that limits microbial growth within the human body (Fischbach *et al*., 2006; Kim and Shin, 2009; Cassat and Skaar, 2013). Based on this, considerable interest has been generated in the possibility of utilising iron chelation therapy as treatment for infectious diseases (Ibrahim *et al*., 2007). However, despite this restriction, successful pathogens have developed strategies such as the use of specific receptors to directly take-up iron from host iron-containing molecules (Sritharan, 2006) and the secretion of iron chelating siderophores (Sritharan, 2006; Hider and Kong, 2010; Vasileva *et al*., 2012). Introduction of high-affinity iron-selective chelating agents will circumvent the methods for iron uptake (Qiu *et al*., 2010) by pathogens as these iron-chelators will compete with microbes for available iron.

Iron chelation therapy involves the use of ligating drugs that ardently bind iron in therapeutic applications (Kalinowski and Richardson, 2005). Iron chelation therapy has predominantly focused on the treatment of iron overload (Hatcher *et al*., 2009) but it is now known that their spectrum of activity extends to the treatment of cancer (Kalinowski and Richardson, 2005), neurodegenerative diseases (Shachar *et al*., 2004; Heli *et al*., 2011) and, potentially, infectious diseases. These ligands promote the excretion and subsequent depletion of iron in biological systems by co-ordinating with intracellular and extracellular iron (Kalinowski and Richardson, 2005). Iron-chelators typically contain nitrogen, oxygen or sulphur-donor atoms that form coordinate bonds with iron (Hatcher *et al*., 2009). These donor atoms affect the preference of the chelator for either the Fe(III) or Fe(II) oxidation states (Kalinowski and Richardson, 2005).

Iron-chelating agents can have either hexadentate, tridentate or bidentate ligands in which six, three or two atoms, respectively, are able to co-ordinate with iron to form octahedral complexes (Liu and Hider, 2002). Iron-chelators with the highest affinity are normally hexadentate and bind iron in a 1:1 ratio (iron: chelator). However, tridentate (1:2 ratio) and bidentate (1:3 ratio) chelators that bind only three or two of the available iron chelation sites can potentially redox cycle and thus

promote generation of free radicals (Hider and Zhou, 2005). This means that incompletely liganded or un-liganded iron ions can react with lipid peroxides or hydrogen peroxide (H_2O_2) in a 'Fenton-type' redox reaction and thus promote generation of lipid radicals or the highly reactive hydroxyl radical (OH•) that can damage nucleic acids, lipid membranes and proteins (Hatcher *et al*., 2009). An iron-chelator must be able to efficiently compete with the biological ligands that normally bind iron to be effective; as a consequence, an iron-chelator's affinity for iron as well as its stoichiometry of iron binding will greatly impact its activity as a therapeutic agent (Buss *et al*., 2003; Buss *et al*., 2004; Hatcher *et al*., 2009).

Importance of the study

Despite the availability of epidemiological data on MRSA in Nigeria, available data are still relatively limited when compared with information from developed countries (Adetayo *et al*., 2014). This can be attributed to several reasons; (a) MRSA is still considered to be a less important cause of morbidity and mortality than other infectious diseases such as malaria, HIV infection and tuberculosis, (b) lack of a national policy for control, screening and reporting of MRSA outbreaks and (c) high cost associated with carrying out molecular studies. Yet *S. aureus*, particularly MRSA, persists as a major cause of nosocomial and community-acquired infections in humans. Findings from this study will therefore provide more insight into the pathogenicity of MRSA from Nigeria.

Aim and objectives of the study

Studies on cases of *S. aureus* infections in travellers returning from Africa have suggested that *S. aureus* isolates from Africa are more virulent than isolates from Europe (Schaumburg *et al*., 2014). In a bid to either confirm or refute this suggestion, this study aims primarily to determine if Nigerian MRSA are more virulent than their UK counterparts. To achieve this, the objectives listed below were set.

1. Confirm the identities of the MRSA isolates under study

The identities of all the isolates as *S. aureus* will be confirmed in PCRs using species-specific thermonuclease (*nuc*) gene primers. Furthermore, primers specific to the *mecA* gene will be used in PCR to confirm methicillin resistance in all the isolates.

2. Characterise the MRSA isolates

A. *Staphylococcus aureus* expresses an array of virulence factors that facilitate attachment to and invasion of host tissues (i.e. surface proteins) and those that cause specific tissue damage (i.e. toxins). Therefore, presence or absence of genes that code for virulence factors known to play roles in tissue attachment, invasion and damage will be probed for in the genomic DNA of all the MRSA isolates using primers specific to the virulence factors in PCRs. The virulence factors selected include surface proteins (*S. aureus* protein A, fibronectin binding proteins A and B), superantigens (enterotoxins A, B, C, D and E, toxic shock syndrome toxin), cytotoxins (panton valentine leucocidin, alpha-, beta-, and gamma toxins), phenol soluble modulins-PSMs (alpha, *mec*, and delta toxin), as well as the types 5 and 8 capsular polysaccharides.

B. As MRSA isolates belonging to the CC22 and 30 lineages are the major clones that dominate clinical UK populations while those belonging to ST239 have been known to cause MRSA infections in Africa, clonal typing of all the isolates (UK and Nigerian) will be done by using primers specific to clonal complexes 22, 30 and ST239 in PCRs.

C. All the MRSA isolates used in this study are of clinical origin, and because MRSA bearing the SCC*mec* types I-III and VIII cause mostly HA-MRSA infections, primers specific to these elements will be used in PCRs to determine the type of SCC*mec* element (Types I-III and VIII in this study) borne by each of the MRSA isolates (UK and Nigerian) under study. In addition, due to the possibility of community-acquired-hospital-onset phenomenon, primers specific to SCC*mec* types IV and V will also be used in PCR to characterise the isolates.

D. Minimum inhibitory concentrations (MICs) are considered the gold standard for determining the susceptibility of organisms to antimicrobial agents (Andrews, 2006). Therefore, the MICs of five antibiotics, from distinct antibiotic classes, (i.e. cefoxitin, vancomycin, gentamicin, ciprofloxacin and tetracycline) against all the MRSA isolates (UK and Nigerian) will be determined.

E. The widely used two-dimensional electrophoresis technique will be used to obtain the protein profiles of the MRSA isolates.

3. Determine the levels at which the UK and Nigerian MRSA isolates interact with mammalian cell lines

The levels of interaction (association, invasion and survival) of MRSA isolates will be ascertained in interaction assays.

4. Determine the cytotoxic effects of UK and Nigerian MRSA isolates on mammalian cell lines

The cytotoxic effects of UK and Nigerian MRSA on mammalian cell lines will assessed in lactate dehydrogenase colorimetric cytotoxicity assays. Toxic effects of the cell-free conditioned medium (medium that has previously supported bacterial growth and thus contains numerous secreted metabolites and signalling molecules endogenous to the particular strain of bacteria) of the UK and Nigerian MRSA on mammalian cells will be assessed in addition to the toxic effect of heat-killed and live MRSA. The contribution of apoptosis and necrosis to the observed cytotoxic effects of live MRSA isolates on the mammalian cells will be assessed using flow cytometry.

5. Determine the anti-MRSA potential of five-novel iron-chelators (iChs)

The limited therapeutic options available for treatment of MRSA infections underline the importance of identifying novel preventive or therapeutic interventions. Therefore, the antimicrobial potential of five novel iron-chelators against MRSA will be assessed.

6. Determine the cytotoxic effect of the five-novel iron-chelators on KB epithelial cell line

This will be assessed in lactate dehydrogenase colorimetric cytotoxicity assays in order to determine the suitability of the iron-chelators for use in topical and systemic human applications.

Chapter Two

Characterisation of the MRSA Isolates under Study

2.1. Introduction

Staphylococcus aureus's capacity to cause a range of important infections in humans is related to the expression of an array of virulence determinants that participate in pathogenesis of infection (Costa *et al*., 2013). These factors can be divided into cell-surface-associated (adherence) and secreted (exotoxins) factors (Costa *et al*., 2013), which are all known to promote tissue colonisation, tissue damage and distant disease (Bien *et al*., 2011). The staphylococcal protein A, fibronectin-binding proteins A/B, collagen-binding protein, clumping factors A/B as well as capsular polysaccharides are examples of the cell-surface-associated virulence determinants expressed by *S. aureus*. These cell-surface-associated virulence determinants play a protective and passive role compared with the secreted virulence determinants which play more active roles in disarming host immunity (Costa *et al*., 2013). Exotoxins disrupt host cells/tissues and also interfere with the host immune system thereby aiding dissemination of bacteria (Foster, 2005; Lin and Peterson, 2010). Types of exotoxins produced by *S. aureus* include cytolytic toxins (e.g α-haemolysin, β-haemolysin, γ-haemolysin, leukocidin, Panton-Valentine leukocidin (PVL) and superantigens (e.g. toxic shock syndrome toxin-1 [TSST-1], the staphylococcal enterotoxins and the exfoliative toxins (ETA and ETB) (Plata *et al*., 2009; Bien *et al*., 2011). The distribution of some virulence factors in *S. aureus* is related to clonal type, whereas the presence of others is unrelated to genetic background (Peacock *et al*., 2002).

The greatest challenge to the treatment of staphylococcal infections is the acquisition of resistance determinants by *S. aureus* because it shows the highest pathogenic potential among the staphylococci (IWG-SCC, 2009). This ability to develop resistance to antibiotics used in its treatment aids the ability of *S. aureus* to cause disease (Omuse *et al*., 2016). SCC*mec* is a mobile genetic element of variable size (range 21 to 67 kb) that is present in MRSA but absent in MSSA (Katayama *et al*., 2000). SCC*mec* essentially carries the *mec* gene complex (which bears the genetic determinant of methicillin reistance: the *mec* gene) in addition to the *ccr* gene complex

(Zong *et al*., 2011). Based on the different classes of the *mec* gene complex and the *ccr* gene types, 13 SCC*mec* elements (I to XIII) have been assigned for *S. aureus* to date [http://www.sccmec.org] (Baig *et al*., 2018). Types I, II III and VIII are often found in healthcare- associated MRSA (HA-MRSA) strains and they carry additional drug-resistance gene(s) while types IV, V and VII are often found in community-acquired MRSA (CA-MRSA) strains and don't carry any additional drug resistance gene(s) (IWG-SCC, 2009; Kuo *et al*., 2012; Namvar *et al*., 2015).

Though β-lactams are the antibiotics of first choice for staphylococcal infections due to their efficiency, increasing MRSA rates and the fact that less than 5% of clinical strains are sensitive to penicillins, means that treatment of *S. aureus* infections now increasingly relies on non-β-lactam antibiotics (Chatterjee and Otto, 2013). Treatment guidelines and surveys indicate that vancomycin is the workhorse of parenteral anti-infective agents for MRSA infections (Rodvold and McConeghy, 2014) even though data from experimental and clinical studies suggest that vancomycin is inferior to β-lactam drugs (Levine *et al*., 1991; Karchmer, 1991). Due to increasing resistance to antimicrobials and strain diversity, treatment of MRSA infections is becoming increasingly more complicated (Chatterjee and Otto, 2013).

The dissemination of specific clones of MRSA has contributed to the accelerated increases in the incidence of MRSA in many parts of the world (Ko *et al*., 2005). Hence, it is important to elucidate the genotypic characteristics of MRSA clones and the genetic relatedness of strains isolated in different geographic regions (Ko *et al*., 2005). Furthermore, precise characterisation of isolates from cases of invasive disease is essential for the design of effective preventive and therapeutic strategies. The purpose of this study was therefore to characterise in some detail the clinical MRSA isolates (UK and Nigerian) to be used during my PhD study. To do this, the presence or absence of selected virulence factors as well as the type of SCC*mec* element (Types I-V and VIII in this study) borne by each of the MRSA isolates employed by this study was investigated using PCR.

Some MRSA clones are spread globally while others are restricted to specific geographical regions (Boswihi *et al*., 2016). For instance, clones of MRSA belonging to the sequence type (ST) 239 lineage are globally distributed (Harris *et al*., 2010; Yamamoto *et al*., 2012) while those belonging to the ST59 and ST93 lineages have limited geographical spread (Boswihi *et al*., 2016). Moreover, though the distribution of MRSA clones in Africa is not well-described, MRSA of the hospital-associated ST239 lineage have been reported in several African countries including Nigeria (Abdulgader *et al*., 2015). On the other hand, clones of the clonal complex (CC) 22 and 30 MRSA lineages are known to predominate in the UK (Knight *et al*., 2012). Therefore, whether the MRSA isolates belonged to the ST239, CC20 or CC30 lineages was investigated in this study. The Kirby-Bauer disc diffusion method is routinely used in clinical laboratories to ascertain the sensitivity of an organism to antibiotics. However, minimum inhibitory concentrations (MICs) are considered the gold standard for determining the susceptibility of organisms to antimicrobial agents and are used to judge the performance of other methods of susceptibility testing (Andrews 2006). Thus, in addition to investigating the susceptibility profile of all the MRSA isolates using the disc diffusion method (to 12 antibiotics), the MICs of five commonly used antibiotics in clinical laboratories against all the isolates was also ascertained.

2.2. Materials and Methods

All chemicals were purchased from Sigma, unless otherwise stated.

2.2.1. Bacterial strains and culture

A total of 22 clinical MRSA isolates, along with MRSA NCTC 12493 and *S. aureus* NCTC 6571 reference strains were used in the course of this study. They were all used in this study because they were confirmed causative agents of diseases (presented in Table 2.1). Six of the 22 MRSA isolates were obtained locally (from Colchester General Hospital, Essex UK), while the other 16 isolates originated from various hospitals in North-eastern Nigeria (Table 2.1). The Nigerian isolates were collected between 2007 and 2012 and have been studied previously (Okon *et al*., 2014), while the UK isolates were collected in 2012. All strains were grown overnight at 37^{o}C on Cysteine Lactose Electrolyte Deficient (CLED) agar under aerobic conditions and refreshed every week. A few single colonies of bacteria were grown under aerobic conditions in Luria-Bertani (LB) broth for 6 hours (hrs) (for antibiotic susceptibility experiments) or overnight (for DNA extraction) at 37^{o}C. In addition, the Optical Density (OD) of each broth culture was measured in a spectrophotometer (Cecil CE 2041) at a wavelength of 595 nm and optical density 0.22. At OD_{595} 0.22, ~10^{8} Colony Forming Units of bacteria per ml [CFU/ml] was obtained.

Table 2.1: Clinical MRSA isolates used during this study and their sources

	ID	Year	Diagnosis	Specimen
UK isolates				
1	16/11	2012	Wound infection	Wound
2	Nasal	2012	Pre-admission screening	Nasal swab
3	Abdo	2012	Wound infection	Wound
4	Shin	2012	Wound infection	Wound
5	2	2012	Wound infection	Wound
6	3	2012	Wound infection	Wound
Nigerian isolates				
1	176	2007	Sepsis	Blood culture
2	113	2007	Conjunctivitis	Eye swab
3	193	2007	Venereal Disease	HVS
4	178	2007	UTI	Urine
5	S43G	2012	UTI	Urine
6	S54N	2012	Surgical wound	Wound
7	158	2007	Sepsis	Wound
8	177	2007	Sepsis	Wound
9	GM12	2012	Wound infection	Wound
10	UM38	2012	Wound infection	Wound
11	UM9	2012	Wound infection	Wound
12	162	2007	Sepsis	Blood culture
13	105	2007	Venereal Disease	HVS
14	112	2007	Venereal Disease	HVS
15	UM44	2012	UTI	Urine
16	114	2007	Sepsis	Wound

An association between disease and *S. aureus* nasal carriage had been noted as far back as 1931 (Wertheim *et al*., 2005) and several studies have confirmed that most *S. aureus* infections originate from strains that colonise the nose (von Eiff *et al*., 2001; Wertheim *et al*., 2005; van Belkum, 2016). Hence the inclusion of UK isolate 'nasal' in this study

2.2.1.1. Justification for use of CLED

Cysteine Lactose Electrolyte Deficient medium supports the growth of all potential urinary pathogens giving good colonial differentiation and clear diagnostic characteristics (Truant 2016, p. 480). Though not as prevalent as organisms such as *Escherichia coli* and *Staphylococcus saprophyticus*, *Staphylococcus aureus* is also a known causative agent of urinary tract infections

(UTIs). Research, like that by Kooi-Pol *et al*., 2013, not carrying out UTI related studies, have used CLED agar to grow *S. aureus*. Furthermore, *S. aureus* is grown on CLED agar in clinical laboratories in the UK. Nevertheless, growth of *S. aureus* on CLED was compared to growth on nutrient agar (which is a non-selective or differential general-purpose medium) to justify its use in this study. The OD of 6-hr cultures of all the MRSA isolates were measured as described in section 2.2.1, to obtain OD_{595} 0.22 (~10^8 CFU/ml). Twenty µl aliquots from diluents of the cultures were then plated onto CLED and NA plates, following which they were incubated at 37^{o}C overnight.

Many researchers found that the productivity of CLED is similar to that of standard procedures (Blood agar and MacConkey agar combined) e.g. the study by Chaturvedi *et al*., 2017.

2.2.2. Extraction of Genomic DNA

Bacterial DNA, for use in polymerase chain reactions, was extracted from all the MRSA isolates (UK and Nigerian) using the QIAamp DNA Mini kit (QIAGEN). Bacteria were harvested from 10 ml of an overnight MRSA culture grown in LB broth and washed twice with phosphate buffered saline-PBS (centrifuged at 8,000 rpm/5009 x g for 5 mins). One hundred and eighty µl of tissue lysis buffer (ATL) and 20 µl of Proteinase K were added to the washed bacteria and mixed before being incubated at 56^{o}C for 1-3 hrs in a water bath. Afterwards, 200 µl of AL lysis buffer (to complete the lysis and help bind the DNA to the spin column) were added to the sample mix which was then further incubated for 10 mins at 70^{o}C in a water bath. Following this, 200 µl of 95% ethanol were added to the sample mix and vortexed for 20 seconds (secs) being transferred into a QIAamp MinElute column. The column was centrifuged at 8,000 rpm/5009 x g for 1 minute (min) and the collection tube discarded and replaced with a new one. Five hundred µl of the first washing buffer (AW1) were added to the spin column and centrifuged for 1 min at 8000 rpm/5009 x g. Five hundred µl of the second washing buffer (AW2) were added to the column to wash the sample a second time (centrifugation for 1 min 8000 rpm/5009 x g) following which the collection tube

was once again discarded and replaced with a new one. The spin column was then spun/centrifuged for 3 min 14,000 rpm/15,339 x g, to dry the membrane. Extracted DNA was collected by adding 50 µl of elution buffer (AE) to the centre of the column membrane, transferring the column into a 1.5 ml eppendorf tube and leaving tube on the bench for 1-5 mins at room temperature. The column was thereafter centrifuged for 1 min at 14,000 rpm/15,339 x g to ensure total collection of the extracted DNA.

2.2.3. Primers

Primers were purchased from Invitrogen as lyophilised pellets and reconstituted to 100 µM (100 pmol/µl) with deionised water. All primers (and their corresponding sequences) used in this study are as shown in Table E.1 (Appendix E). However, all the primers were used in monoplex PCRs rather than in multiplex PCRs as described in some of the references. The PCR primers were chosen based on their specificity to the target genes. Therefore, since they were not used in multiplex reactions, the PCR cycling parameters were modified accordingly to optimise specific DNA amplification.

2.2.4. Polymerase chain reactions (PCRs)

Taq DNA polymerase (Life technologies) and DreamTaq DNA polymerase (Life technologies) were used for PCR amplification of specific regions of DNA. PCR mix for each monoplex reaction contained:

1. 100 ng genomic DNA;
2. 0.4 µM of each primer;
3. 1.5 mM $MgCl_2$;
4. 1X Taq buffer;
5. 200 µM dNTPs; and
6. 1 unit Taq DNA polymerase.

Each mix was made up to 25 µl with deionised water. All amplifications were done in an EdvoCycler thermal cycler (Edvotek Inc.) using cycling parameters described in the Table E.2 (Appendix E).

2.2.5. Agarose gel electrophoresis

PCR products were analysed in 1% or 1.5% agarose gels (as necessary) to which 3 µl of SafeView nucleic acid stain (nbs biologicals) had been added per 100 ml (of agarose gel) for fluorescent visualisation of DNA bands. Samples for electrophoresis were prepared by adding one volume of 6X DNA Loading Dye (Thermo Scientific) to five volumes of PCR sample to be analysed. Gels were run in 1X TAE electrophoresis buffer at 150V for 15-30 mins in a Sub-gel midi-plus gel electrophoresis apparatus (Fisher). Gels were visualised in a Syngene InGenius^3 gel documentation system.

2.2.6. Antibiotic Susceptibility

2.2.6.1. Antibiotic disc diffusion test

The susceptibility of all the MRSA isolates used during this study (all six UK and 16 Nigerian isolates) to 12 antibiotics was determined using the disc diffusion (Kirby-Bauer) method, The antibiotics used include; Cefoxitin (30 µg), Linezolid (10 µg), Fusidic acid (10 µg), Tetracycline (30 µg), Clindamycin (2 µg), Ciprofloxacin (5 µg), Amikacin (30 µg), Erythromycin (15 µg), Tigecycline (15 µg), Quinupristin/Dalfopristin (15 µg), Rifampicin (5 µg) and Gentamicin (10 µg). A 6-hr broth culture of each isolate was adjusted to a McFarland 0.5 turbidity standard (~1-2 x10^8 CFU/mL). Sterile swab sticks were dipped into each bacterial suspension and swabbed on the entire surface of Mueller Hinton agar plates. Plates were left to dry for about 5 mins following which antibiotic discs were placed on the surface of the agar plates. All plates were then incubated at 37^{o}C for 18 hrs. The zones of inhibition around the antibiotic discs were measured and the

results interpreted as resistance or susceptible based on the interpretative standard of the European Committee on Antibiotic Susceptibility Testing - EUCAST (EUCAST, 2017).

2.2.6.2. Minimum inhibitory concentration (MIC) micro-dilution assays

Minimum inhibitory concentration micro-dilution assays were performed to determine the inhibitory effect of five antibiotics, commonly used in clinical laboratories (i.e. gentamicin, ciprofloxacin, cefoxitin, vancomycin and tetracycline), against all the MRSA isolates (UK and Nigerian) under study. These five antibiotics were selected on the basis that they were effective against Gram-positive bacteria and belonged to different antibiotic classes i.e. cefoxitin (beta-lactam), tetracycline, vancomycin (glycopeptide), ciprofloxacin (fluoroquinolone) and gentamicin (aminoglycoside). One or two single colonies of each MRSA isolate were grown in LB broth for 6 hrs at 37^{o}C under aerobic conditions. The OD of the cultures were measured as described in section 2.2.1.

All assays were performed in 96-well plates with ~10^5 CFU/ml of bacteria per well in the presence of different concentrations (ranging from 0.75 – 32 µg/ml) of antibiotics. To get accurate concentrations of antibiotic per well, antibiotics were prepared/diluted with deionised water. To get ~10^5 CFU/ml of bacteria per well, 6-hour broth culture of each isolate with known number of CFU (i.e. OD_{595} 0.22, ~10^8 CFU) were first diluted with PBS, following which an appropriate volume from the dilution was added to each well containing a specific amount of antibiotic. Antibiotic free controls were also set up in this experiment. Plates were then incubated overnight (typically about 18 hours) under aerobic conditions at 37^{o}C. The number of viable bacteria in each well was determined by plating 20 µl (in duplicate) of the content of each well onto CLED agar. The number of colonies on agar was counted on a colony counter (Gallenkamp) after overnight incubation at 37^{o}C. Bacterial inhibition rate was calculated as follows:

$$(R = \frac{[X_0 - X_t]}{X_0} \times 100)$$

where R is the bacterial inhibition rate, X_0 the number of bacteria without antibiotic (control), and X_t the number of bacteria after the treatment with antibiotic. The MIC is the lowest concentration of an antimicrobial agent that completely visually inhibits 99% growth of the microorganisms after overnight incubation (Ansari *et al*., 2011; Sultan *et al*., 2015).

2.3. Results

2.3.A. Growth of MRSA on CLED agar

In order to justify the use of CLED in this work, growth of MRSA on CLED was compared with growth on nutrient agar. The main differences observed were with regard to pigmentation of colonies formed and pigmentation of medium after growth. While the colonies on CLED agar were seen to be deep yellow in colour (a function of the ability of all the isolates to ferment lactose present in CLED agar), the colonies on nutrient agar were white in colour (Figure 2B). Furthermore, the CLED media were seen to change from the original green colour to light yellow following growth of MRSA while no change was observed in the colour of the nutrient agar medium (Figure 2B). With regard growth of viable colonies, the results showed that growth of MRSA on CLED was similar to growth of the isolates on nutrient agar (Figure 2A). Statistical analysis of data using the two-tailed unpaired t-test showed that the differences observed in number of colony forming units of MRSA on CLED and nutrient agar were insignificant (*P*-value <0.05 i.e. 0.85)

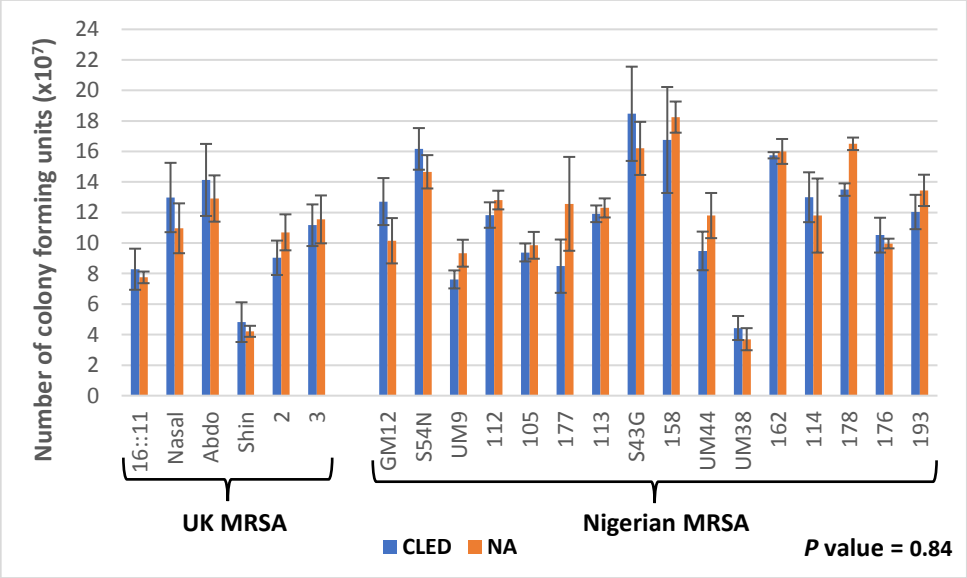


Figure 2A: Plot showing growth of MRSA on CLED and nutrient agar. Results are the mean of three independent experiments; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired two-tailed t test assuming equal variance.

Nutrient agar CLED

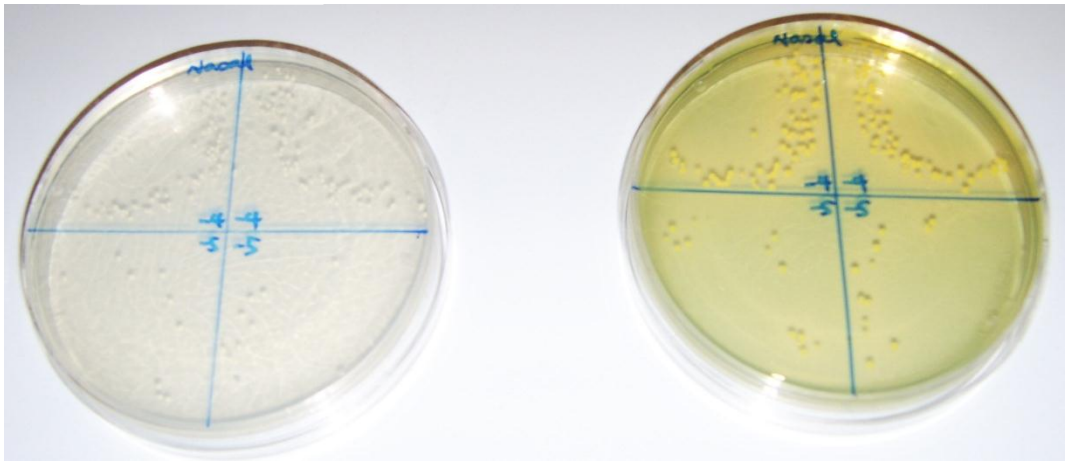


Figure 2B: Picture showing growth of an MRSA isolate on nutrient agar and CLED. Twenty µl aliquots of 10^{-4} (-4) and 10^{-5} (-5) dilutions of an overnight culture of UK isolate Nasal were plated (in duplicate) on nutrient agar and CLED.

2.3.1. Identities of the MRSA isolates under study

2.3.1.1. PCR amplification of the *S. aureus* Thermonuclease (nuc) gene

A 270 bp region of DNA specific to the nuc gene of *S. aureus* species was amplified from the genomic DNA of all the 22 UK and Nigerian MRSA isolates (gel pictures as seen in Figure 2.1) using primers, nuc 1 and nuc 2 (gel pictures as seen in Figures 2.1 and D.1-Appendix D). All the MRSA isolates were therefore confirmed to be *S. aureus*. The nuc specific DNA was also amplified in both the MRSA NCTC 12493 and *S. aureus* NCTC 6571 reference strains.

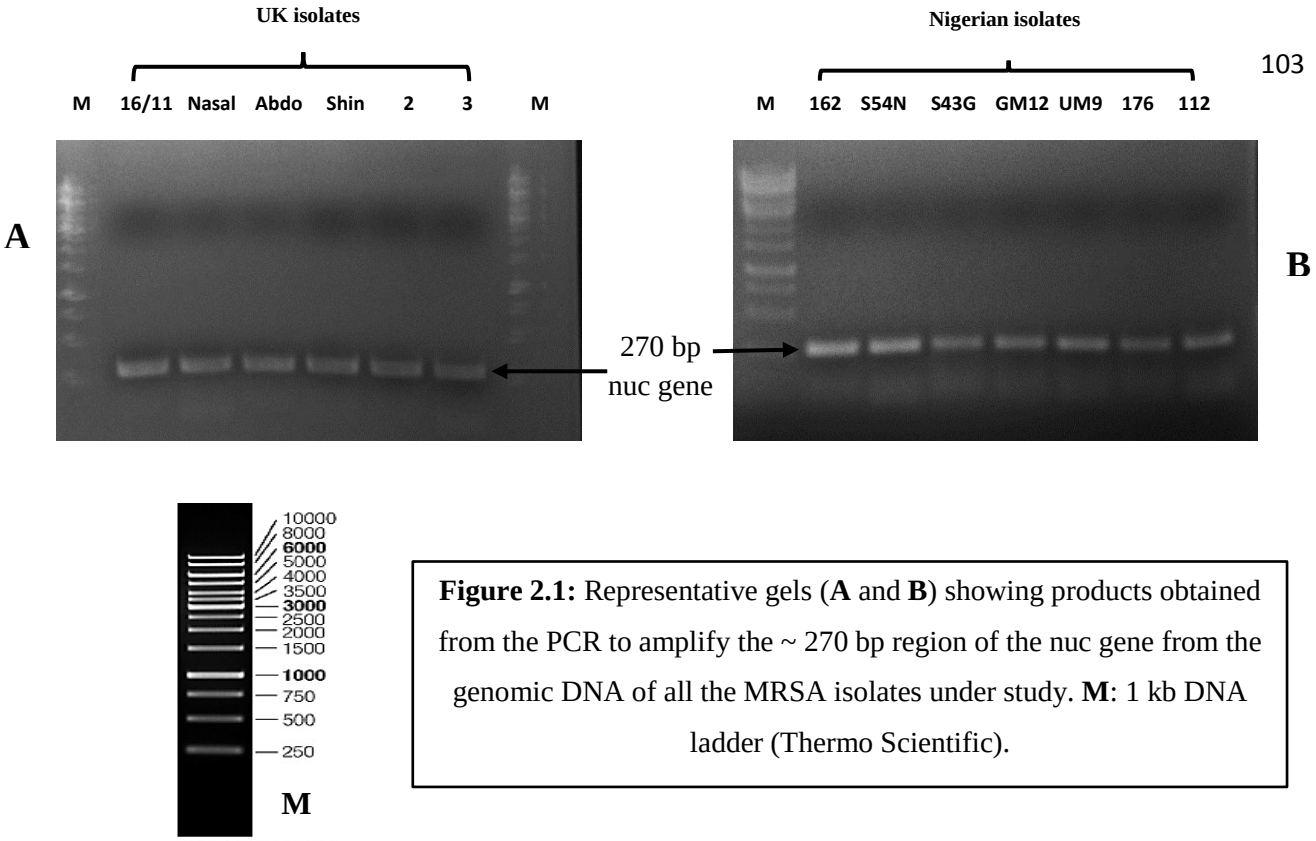


Figure 2.1: Representative gels (**A** and **B**) showing products obtained from the PCR to amplify the ~ 270 bp region of the nuc gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).

2.3.1.2. PCR amplification of the MRSA *mec*A gene

Methicillin resistance was confirmed in all 22 UK and Nigerian MRSA isolates due to the PCR amplification of a 500 bp *mec*A specific DNA from their genomic DNA using primers mec 1 and mec 2 (gel pictures as seen in Figures 2.2 and D.2-Appendix D). As expected, the *mec*A gene was present in the MRSA NCTC 12493 reference strain and was absent in the *S. aureus* NCTC 6571 reference strain (Figures 2.2 and D.2-Appendix D).

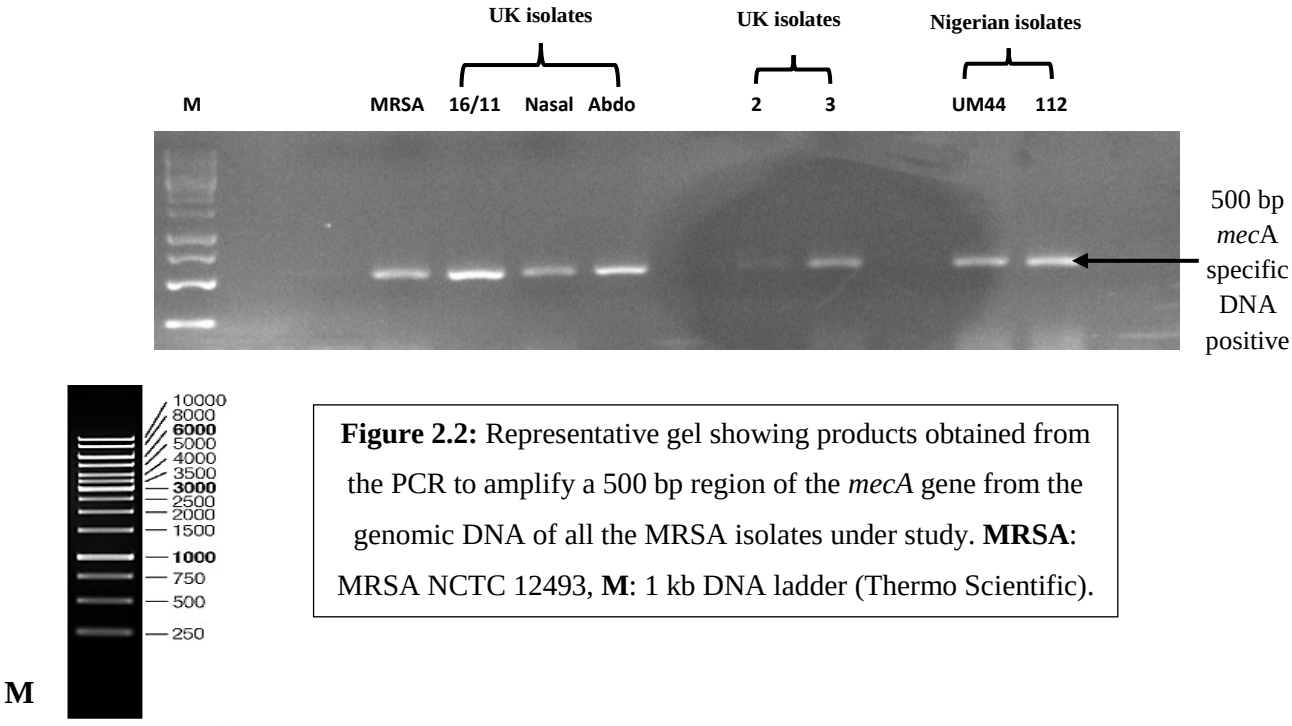


Figure 2.2: Representative gel showing products obtained from the PCR to amplify a 500 bp region of the *mecA* gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **M**: 1 kb DNA ladder (Thermo Scientific).

2.3.2. SCC*mec* typing

Primers specific to Class A and B *mec* gene complexes as well as those specific to the types 1, 2 and 3 cassette chromosome recombinase (*ccr*) gene complexes (all listed in Table 2.2) were used in PCR to ascertain their presence or absence in all 22 MRSA isolates (UK and Nigerian) under study. Furthermore, primers specific to SCC*mec* types IV and V (listed in Table 2.2) were also used in PCR to determine if any of the MRSA isolates bear the said SCC*mec* types.

Based on the combination of the amplified *mec* and *ccr* gene complexes from the genomic DNA of the MRSA isolates (gel pictures as seen in Figures 2.3 – 2.8 and Figures D.3 – D.7 [Appendix D]) and the IWG-SCC guidelines (www.sccmec.org), the results show that two of the MRSA isolates (one UK – 16.67% and one Nigerian – 6.25%) bear the SCC*mec* type II while only one UK isolate (UK isolate 'Shin') bears the SCC*mec* type I. Furthermore, nine (56.25%) of the Nigerian isolates were seen to bear the SCC*mec* type III. As in the studies by Boyle-Vavra *et al*., 2005, Hanssen *et al*., 2007 and Chen *et al*., 2017, more than one *ccr* gene complex was amplified in some of the isolates (three UK MRSA isolates [50%] and two Nigerian MRSA isolates [12.5%]) (Table 2.2). Therefore, these isolates could not be classified based on the typing system used in this study. Also, the SCC*mec* types borne by isolates GM12 (UK isolate) and UM44 (Nigerian isolate) could not be identified based on the primers used in this study. Additionally, no classification was found for UK isolate 16/11 because it bears the class B *mec* gene complex and the type 3 *ccr* gene complex and as yet, there is no grouping for this SCC*mec* type based on the IWG-SCC guidelines on the sccmec.com website.

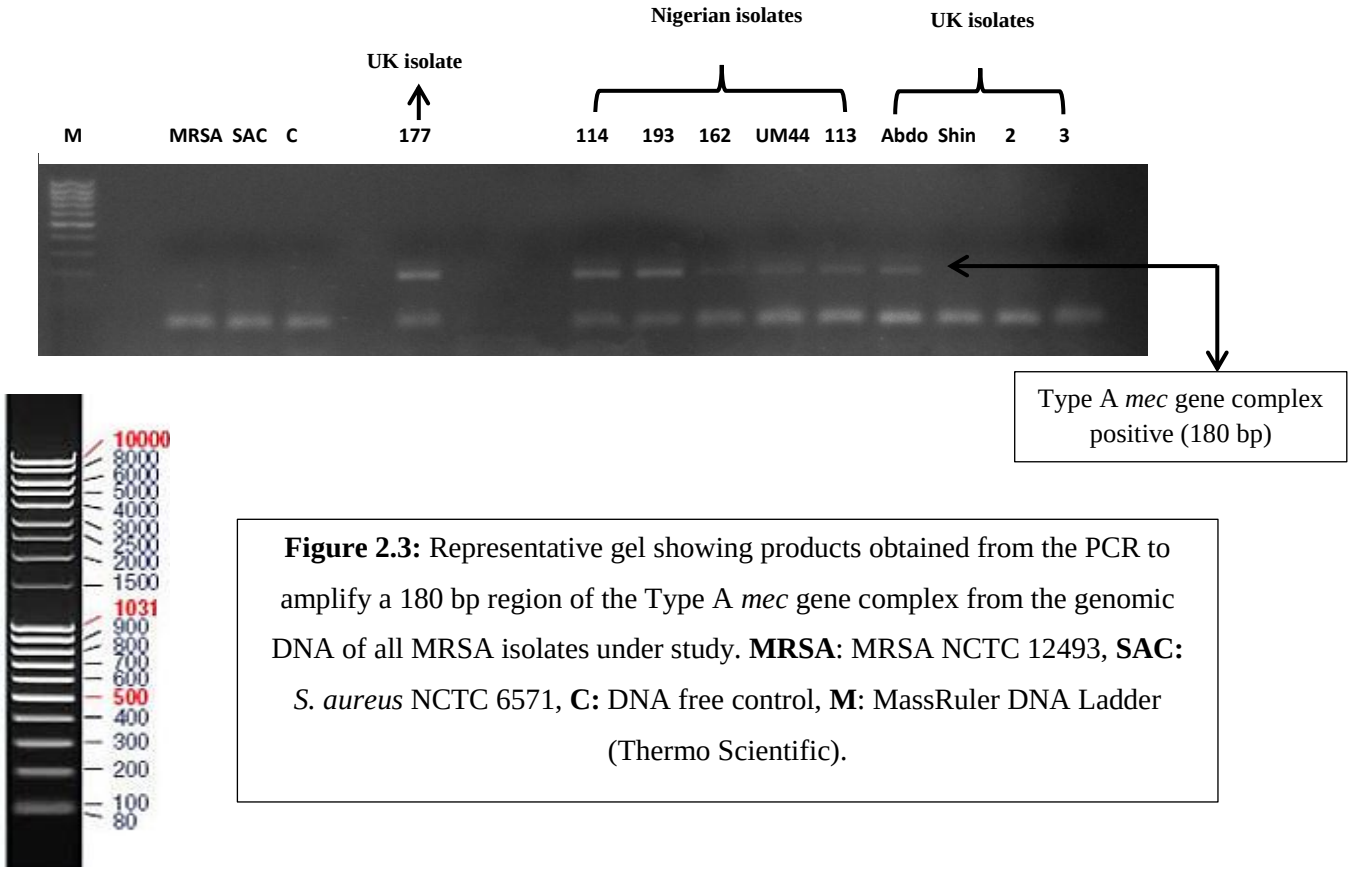


Figure 2.3: Representative gel showing products obtained from the PCR to amplify a 180 bp region of the Type A *mec* gene complex from the genomic DNA of all MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: MassRuler DNA Ladder (Thermo Scientific).

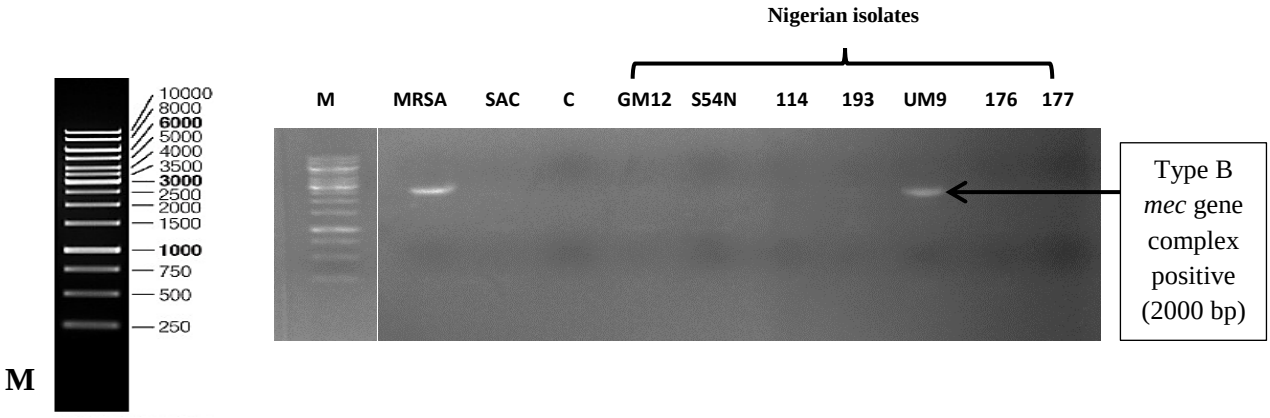
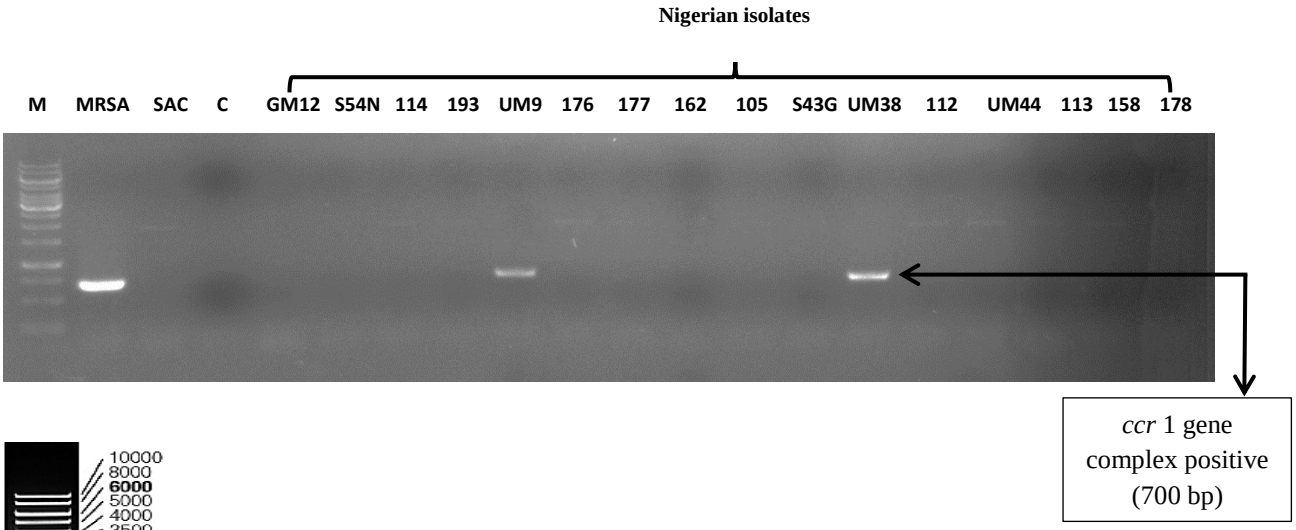


Figure 2.4: Representative gel showing products obtained from the PCR to amplify a 2000 bp region of the Type B *mec* gene complex from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

Even though PCR products from the amplification of the Type B *mec* gene (from the genomic DNA of all the isolates) were run on several gels, the amplified products were still seen to be closer to 2500 bp rather than the 2000 bp band marker on the DNA ladder. This could be a function of the type DNA ladder that was used.



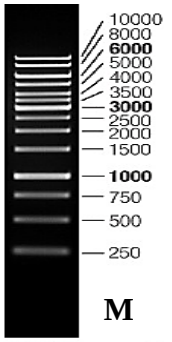


Figure 2.5: Representative gel showing products obtained from the PCR to amplify a 700 bp region of the Type 1 *ccr* gene complex from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

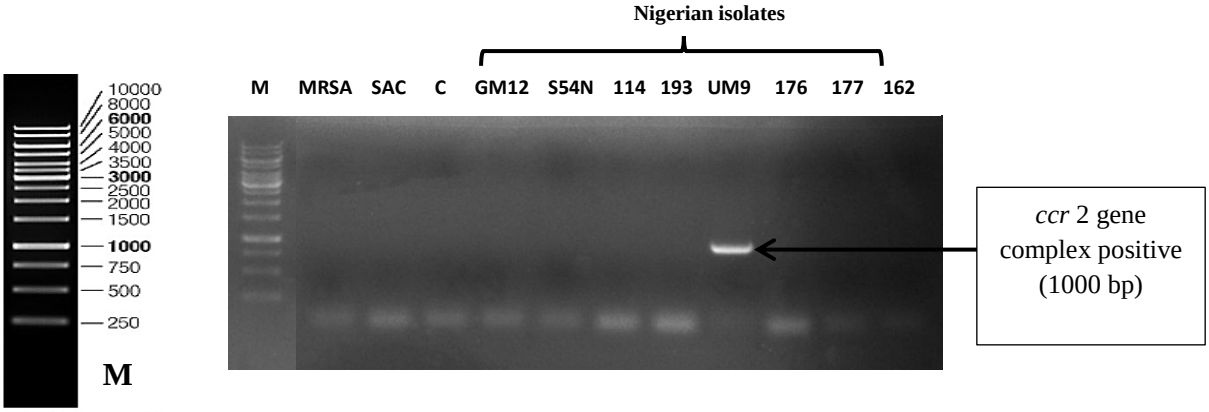


Figure 2.6: Representative gel showing products obtained from the PCR to amplify a 1000 bp region of the Type 2 *ccr* gene complex from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

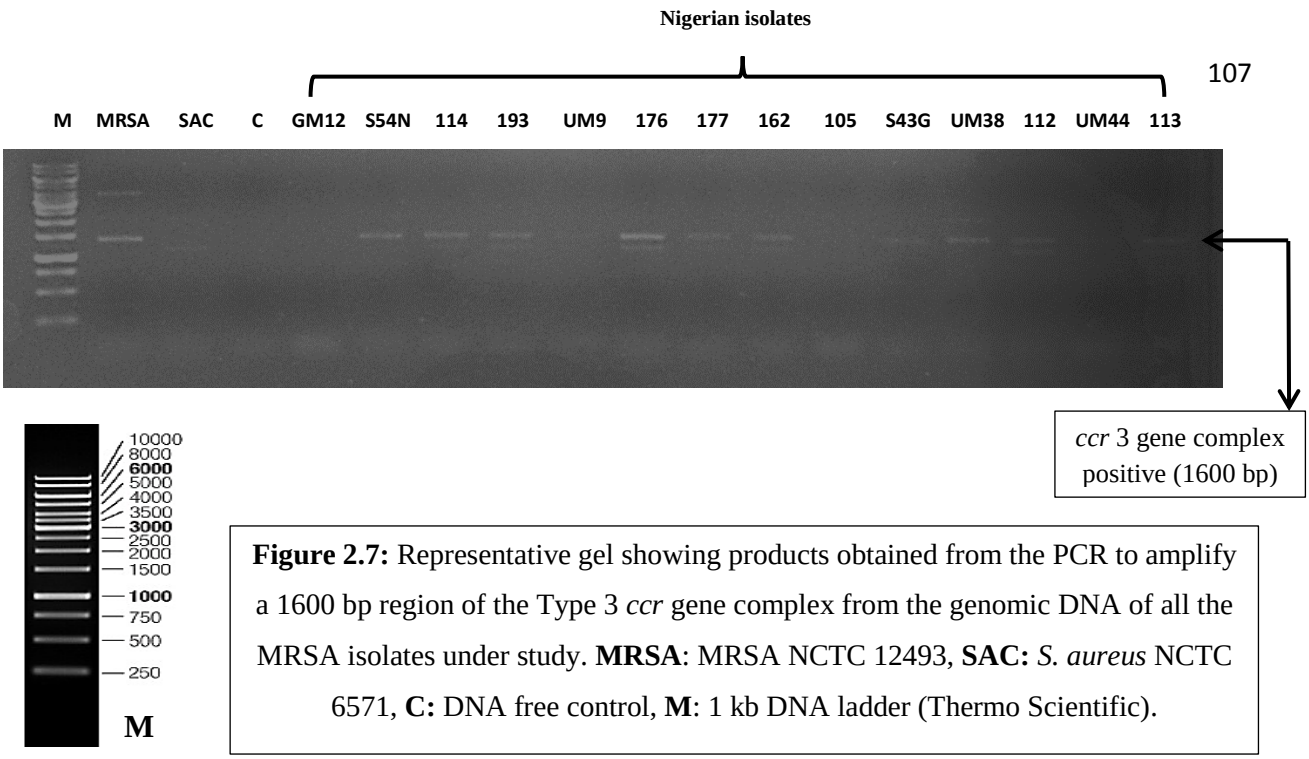


Figure 2.7: Representative gel showing products obtained from the PCR to amplify a 1600 bp region of the Type 3 *ccr* gene complex from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

SCC*mec* type V was found to be borne by isolates S54N and S43G based on the use of primers specific to a 325 bp region specific to SCC*mec* type V. DNA fragments specific to SCC*mec* type IV subtypes (subtypes A-F: Primers as listed in Table E.1 [Appendix E]) was not amplified from the DNA of any of the MRSA isolates (UK or Nigerian) [Gel pictures not shown].

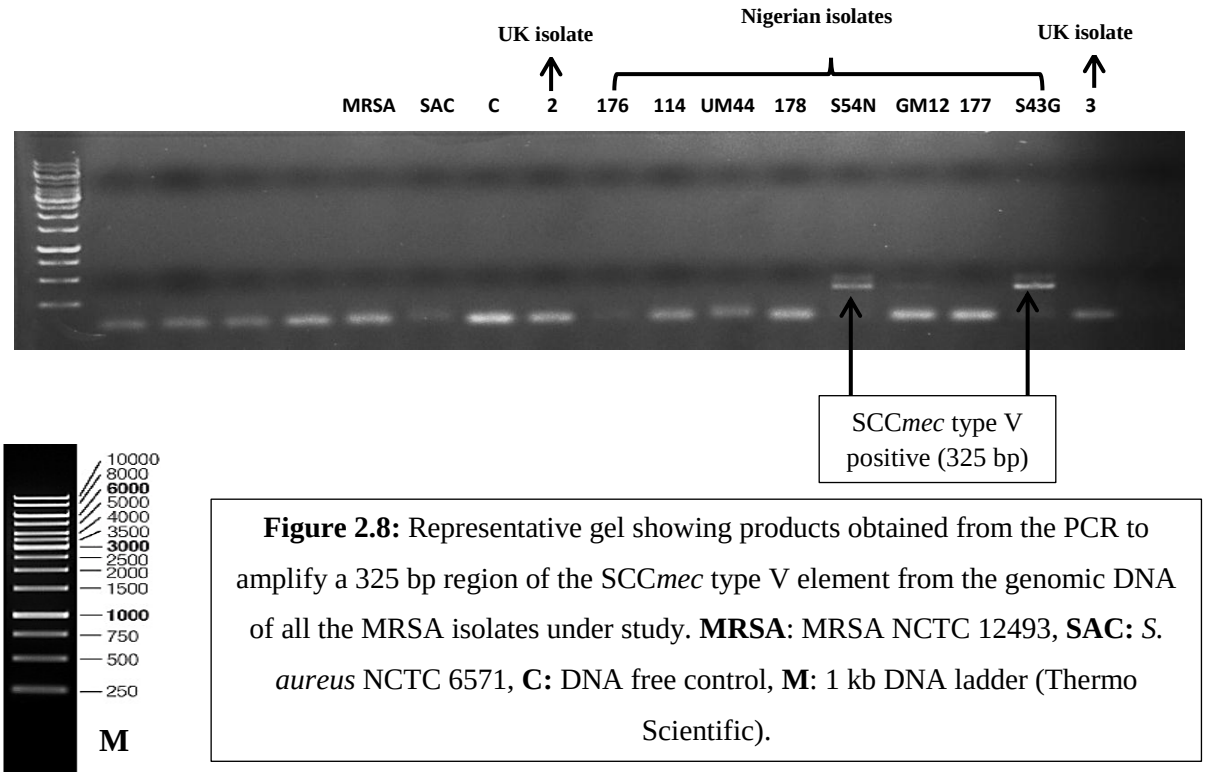


Figure 2.8: Representative gel showing products obtained from the PCR to amplify a 325 bp region of the SCC*mec* type V element from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

Results obtained from the PCRs to confirm the identities of all the UK and Nigerian MRSA isolates as well as those done to determine the SCC*mec* types borne by the isolates are as summarised in Table 2.2.

Table 2.2 Summary of results from PCRs to confirm the identities of the MRSA isolates as well as results from the SCC*mec* typing of the isolates

Isolate	nuc gene	*mec* A gene	*mec* gene complex		*ccr* gene complex			SCC*mec* type
			Class A	Class B	Type 1	Type 2	Type 3	
UK								
16/11	+	+		+			+	
Nasal	+	+		+	+	+		
Abdo	+	+	+			+		**II**
Shin	+	+		+	+			**I**
2	+	+		+	+	+		
3	+	+		+	+	+		
Total (%)	**100**	**100**	**16.67**	**83.33**	**66.67**	**66.67**	**16.67**	
Nigerian								
GM12	+	+						
S54N	+	+					+	**V**
114	+	+	+				+	**III**
193	+	+	+				+	**III**
UM9	+	+		+	+	+		
176	+	+	+				+	**III**
177	+	+	+				+	**III**
162	+	+	+				+	**III**
105	+	+	+			+		**II**
S43G	+	+					+	**V**
UM38	+	+		+	+	+	+	
112	+	+	+				+	**III**
UM44	+	+	+					
113	+	+	+				+	**III**
158	+	+	+				+	**III**
178	+	+	+				+	**III**
Total (%)	**100**	**100**	**68.75**	**12.5**	**12.5**	**18.75**	**75**	
***S. aureus* NCTC 6571**	+							
MRSA NCTC 12493	+	+		+	+		+	

2.3.3. PCR amplification of virulence factors

Specific primers, as listed in Table E.1 (Appendix E), were used in PCRs to ascertain the presence of genes that encode selected virulence factors i.e. *S. aureus* protein A (*spa*), toxic shock syndrome toxin (*tst*), phenol soluble modulins (PSMα and δ toxin), haemolysins (α, β, and γ toxins) and PVL, fibronectin binding proteins A and B, enterotoxins A, B, C, D and E, as well as the types 5 and 8 capsular polysaccharides.

Results obtained show that all the MRSA isolates (both UK and Nigerian) bear both the *spa* gene and fibronectin-binding protein A (*fnbA*) gene (Figures 2.9 and 2.10 respectively).

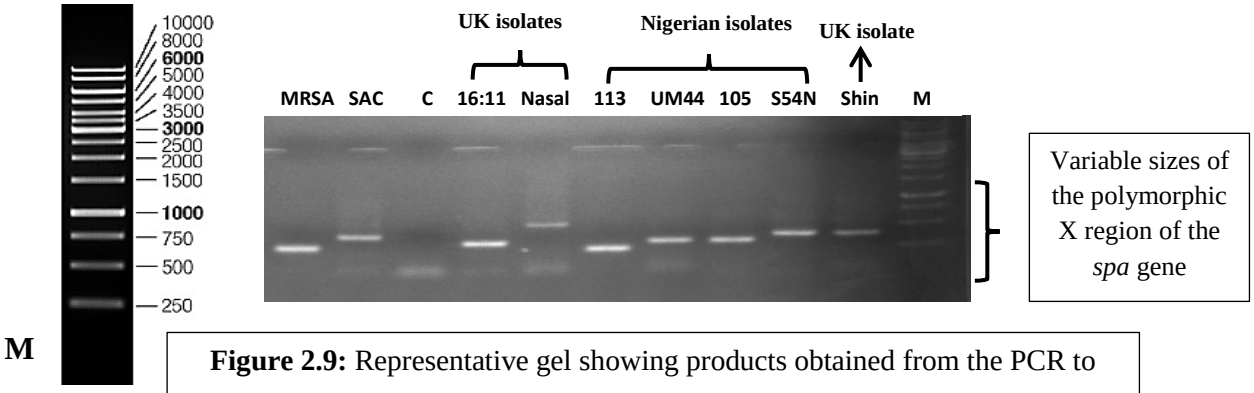


Figure 2.9: Representative gel showing products obtained from the PCR to amplify the polymorphic X region of the *spa* gene from the genomic DNA of all MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

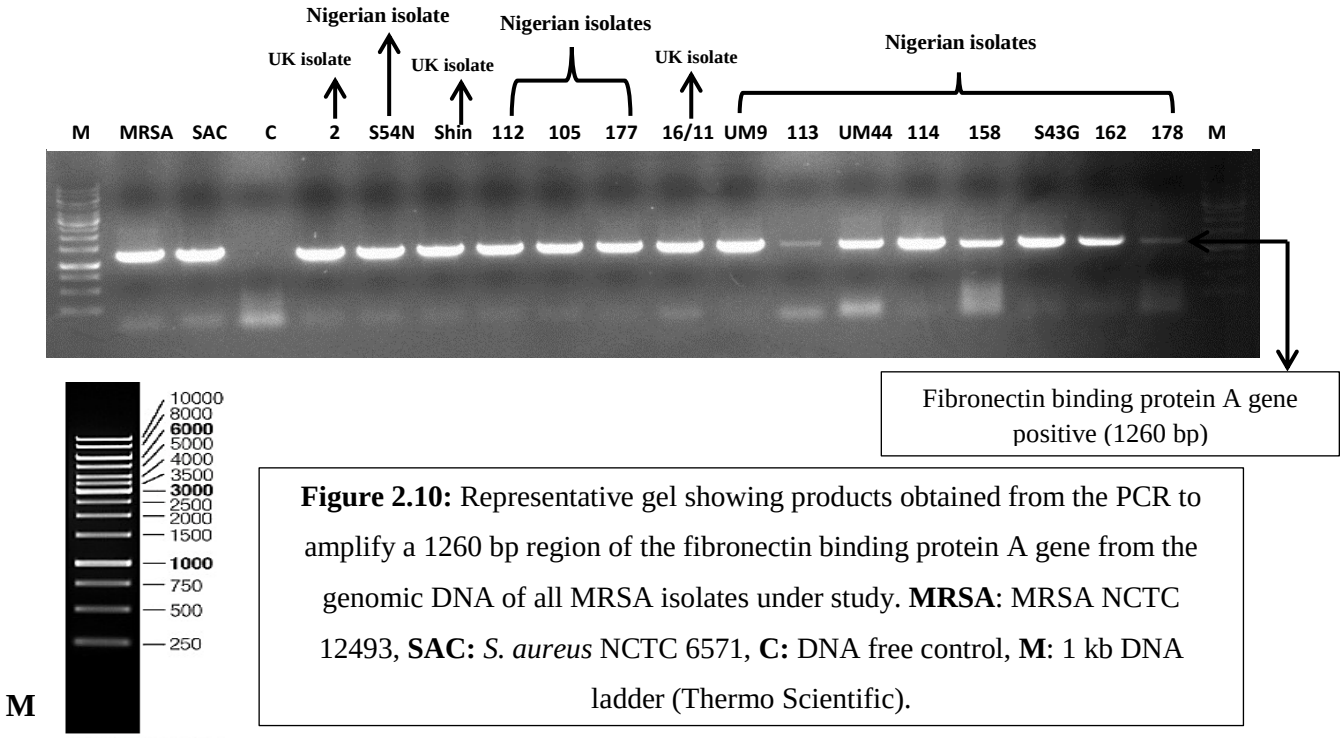


Figure 2.10: Representative gel showing products obtained from the PCR to amplify a 1260 bp region of the fibronectin binding protein A gene from the genomic DNA of all MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

While none of the UK isolates were positive for enterotoxin B gene, it was detected in two (12.5%) of the Nigerian MRSA isolates i.e. S43G and S54N (Figures 2.11 and D.21-Appendix D).

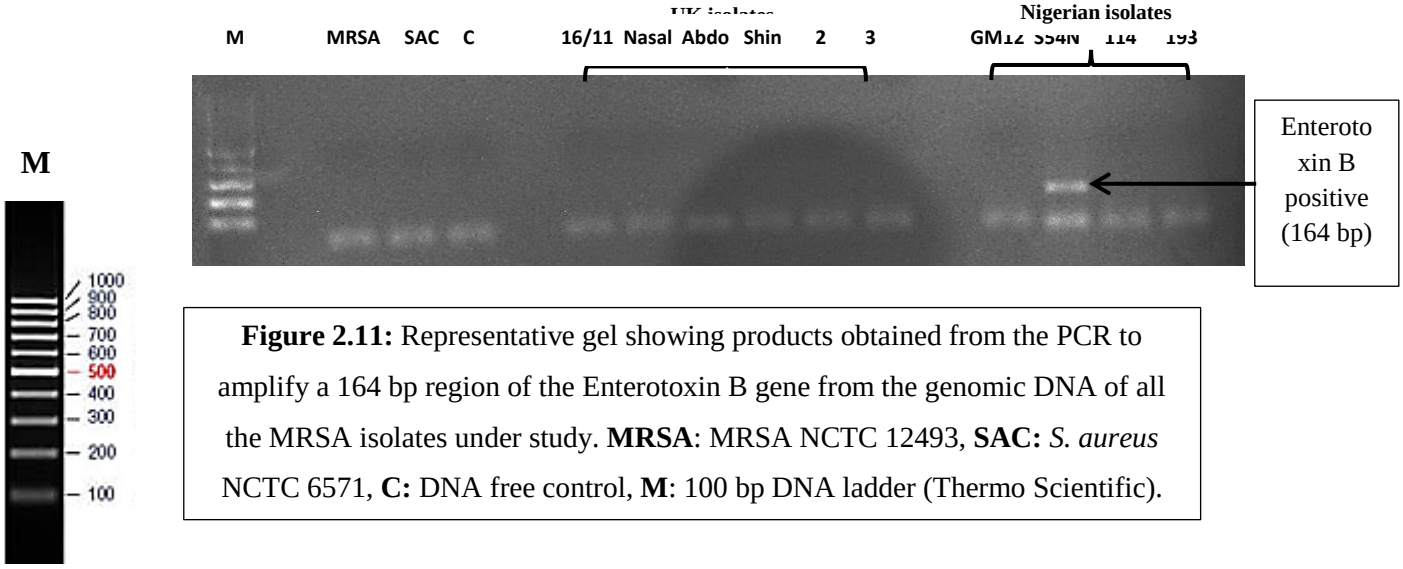


Figure 2.11: Representative gel showing products obtained from the PCR to amplify a 164 bp region of the Enterotoxin B gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 100 bp DNA ladder (Thermo Scientific).

The enterotoxin C gene was seen to be present in only three (50%) of the UK isolates (16/11, Nasal, 2) and in three (18.75%) of the Nigerian MRSA isolates (162, 178, 177) as well (Figures 2.12 and D.10 [Appendix D]).

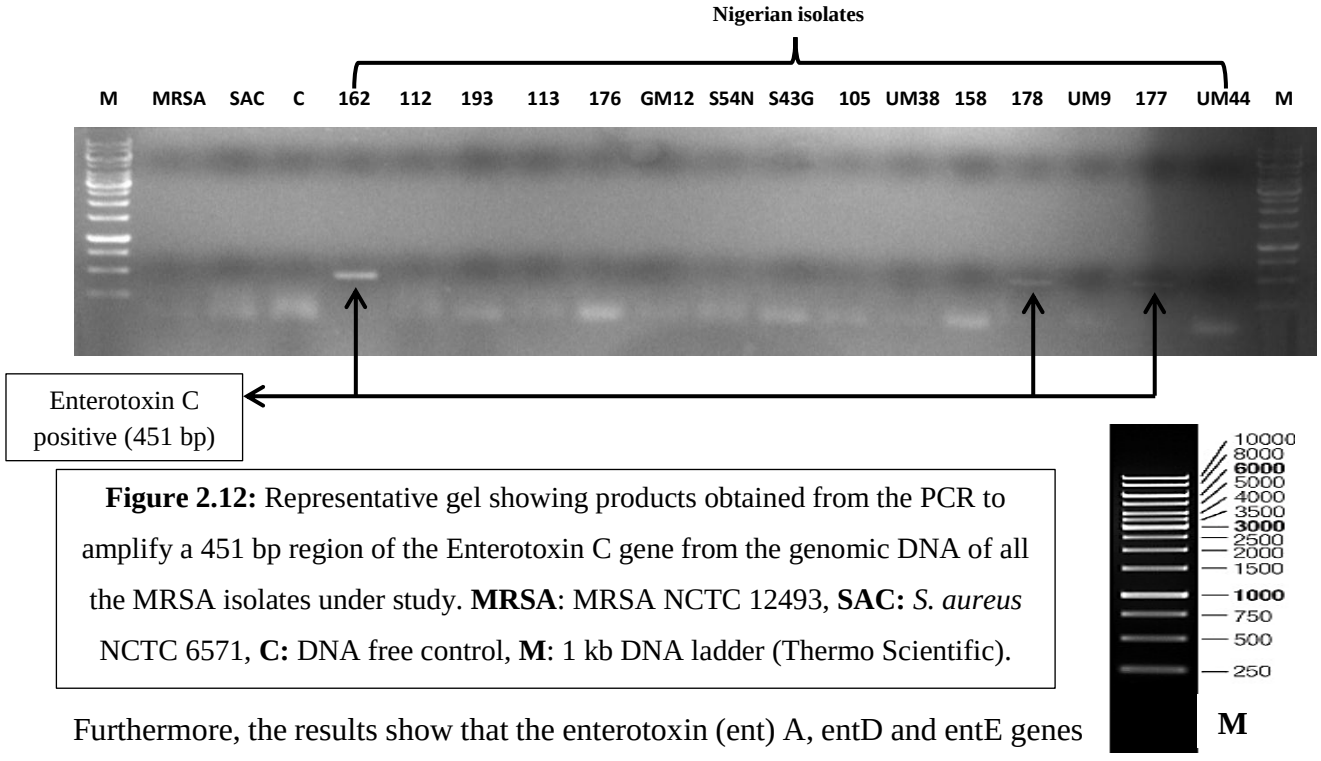


Figure 2.12: Representative gel showing products obtained from the PCR to amplify a 451 bp region of the Enterotoxin C gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

Furthermore, the results show that the enterotoxin (ent) A, entD and entE genes any of the MRSA isolates (both UK and Nigerian) (Gel pictures not shown).

Only two (33.33%) UK MRSA isolates (Shin, Abdo) were positive for the *tst* gene while all of the Nigerian MRSA isolates were negative for the *tst* gene (Figures 2.13 and D.11 [Appendix D]).

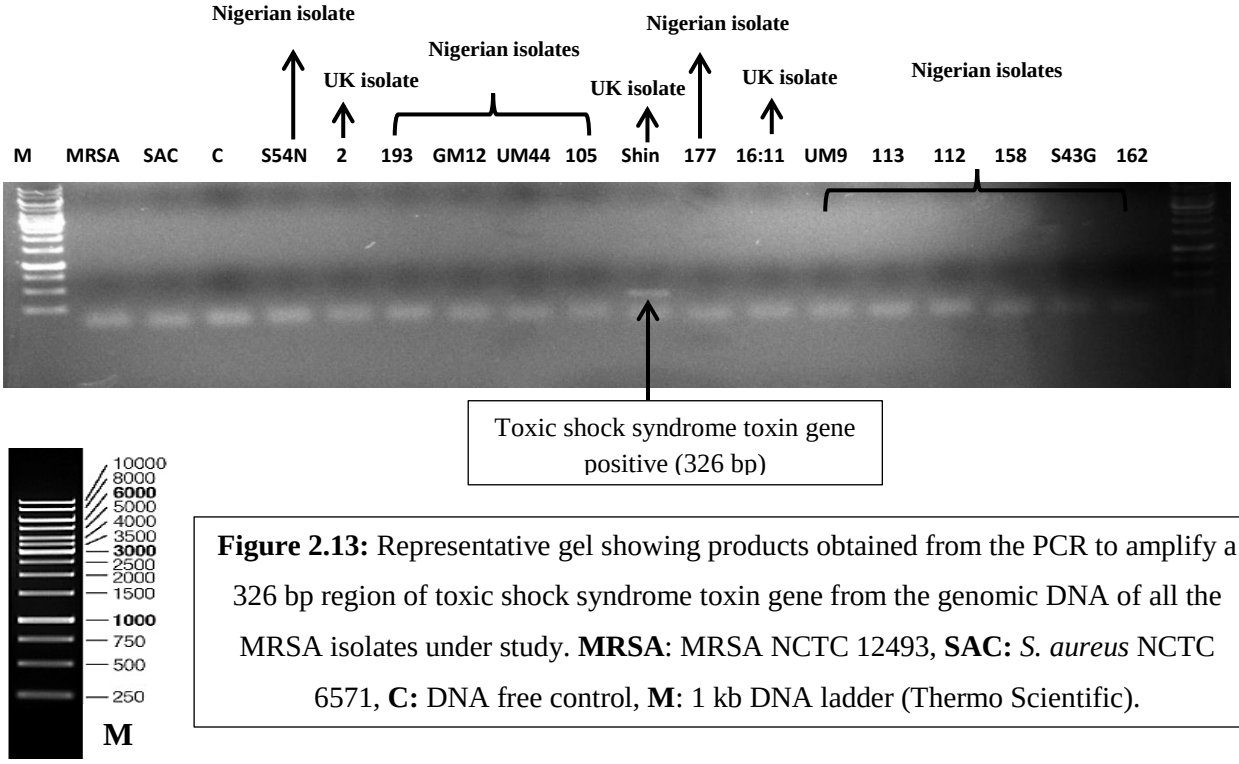
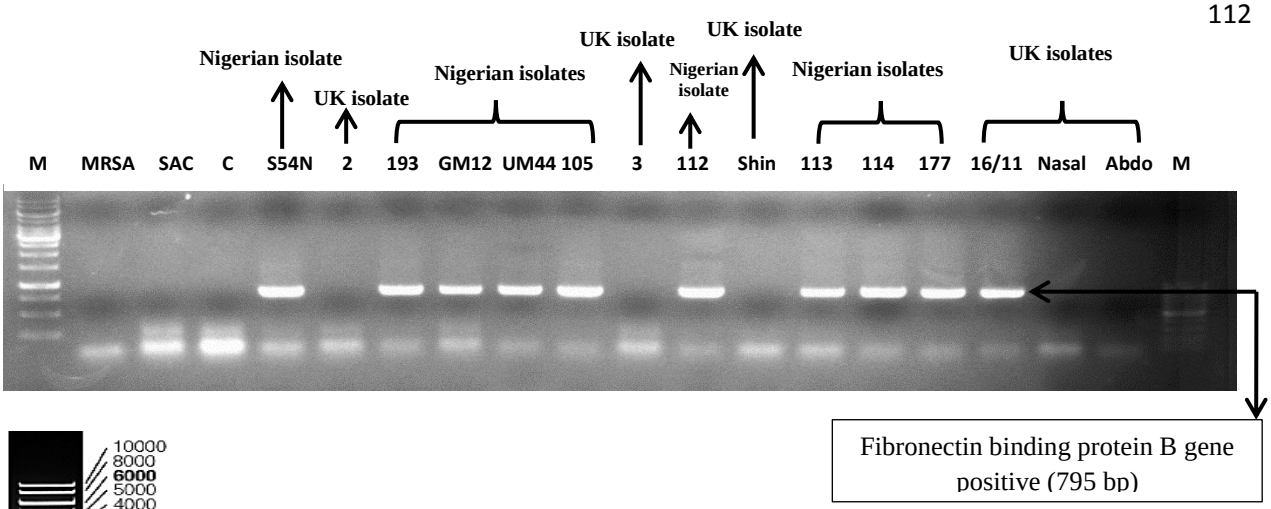


Figure 2.13: Representative gel showing products obtained from the PCR to amplify a 326 bp region of toxic shock syndrome toxin gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

The fibronectin-binding protein B gene (*fnbB*) was seen to be present in only one (16.67%) UK isolate (16/11) while it was seen to be absent in only two (12.5%) of the Nigerian isolates (UM9, UM38) (Figures 2.14 and D.12 [Appendix D]).



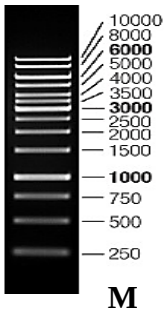
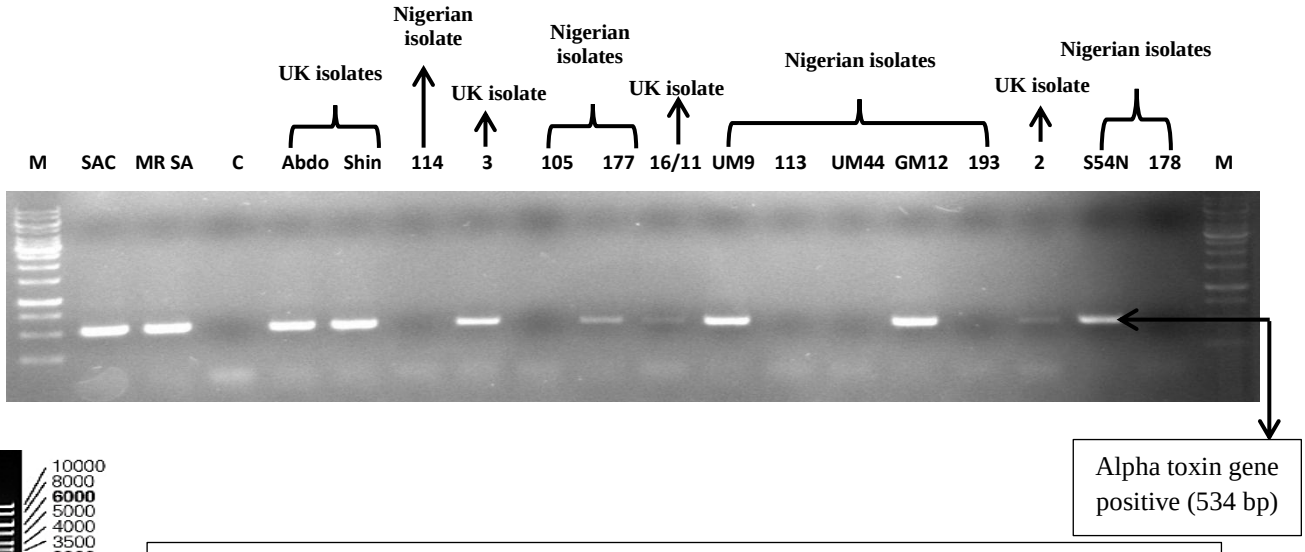


Figure 2.14: Representative gel showing products obtained from the PCR to amplify a 795 bp region of the fibronectin binding protein B gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

All the UK isolates (100%) were seen to be positive for the α-toxin/haemolysin (*hla*) gene while only 50% of the Nigerian isolates bear the *hla* gene (Figures 2.15 and D.13 [Appendix D]).



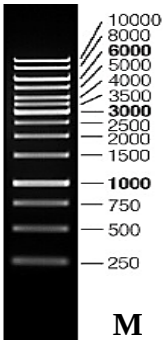


Figure 2.15: Representative gel showing products obtained from the PCR to amplify a 534 bp region of alpha toxin gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

In contrast, only 50% of the UK isolates (16/11, Shin, 2) bear the β-toxin/haemolysin (*hlb*) gene while all the Nigerian isolates (100%) were seen to bear the β-toxin gene (Figures 2.16 and D.14 [Appendix D]).

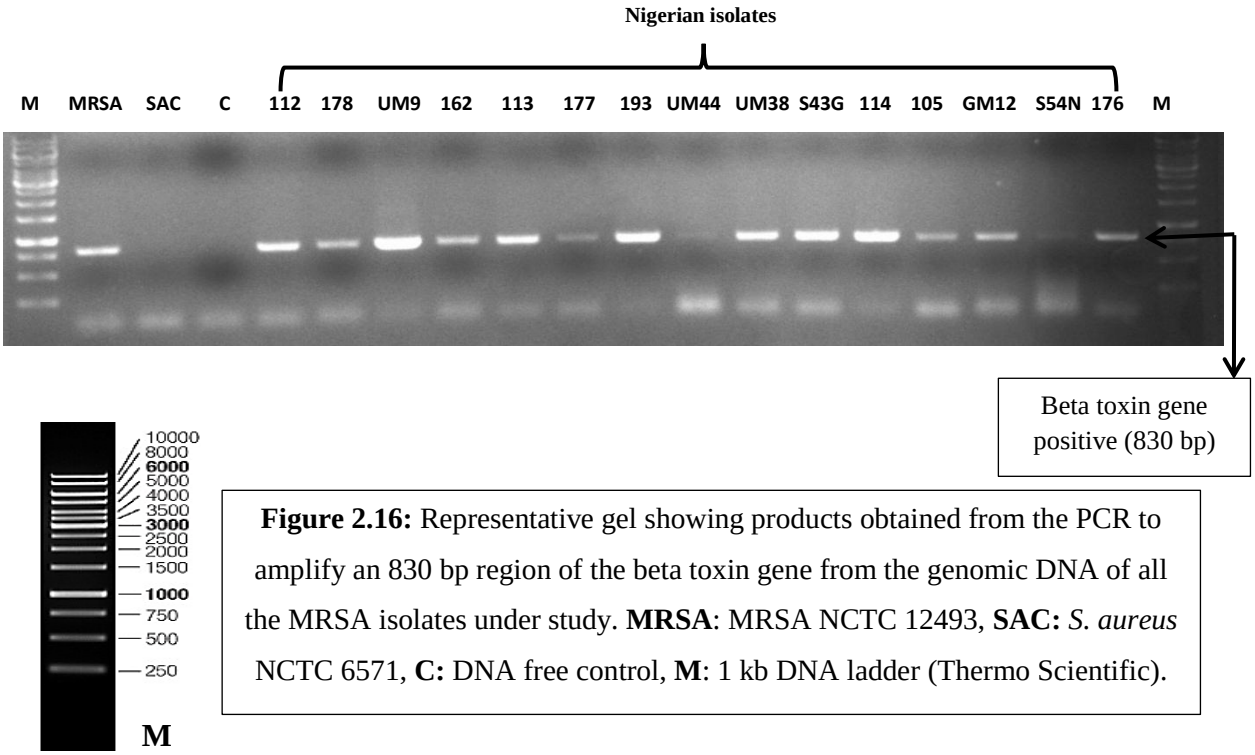
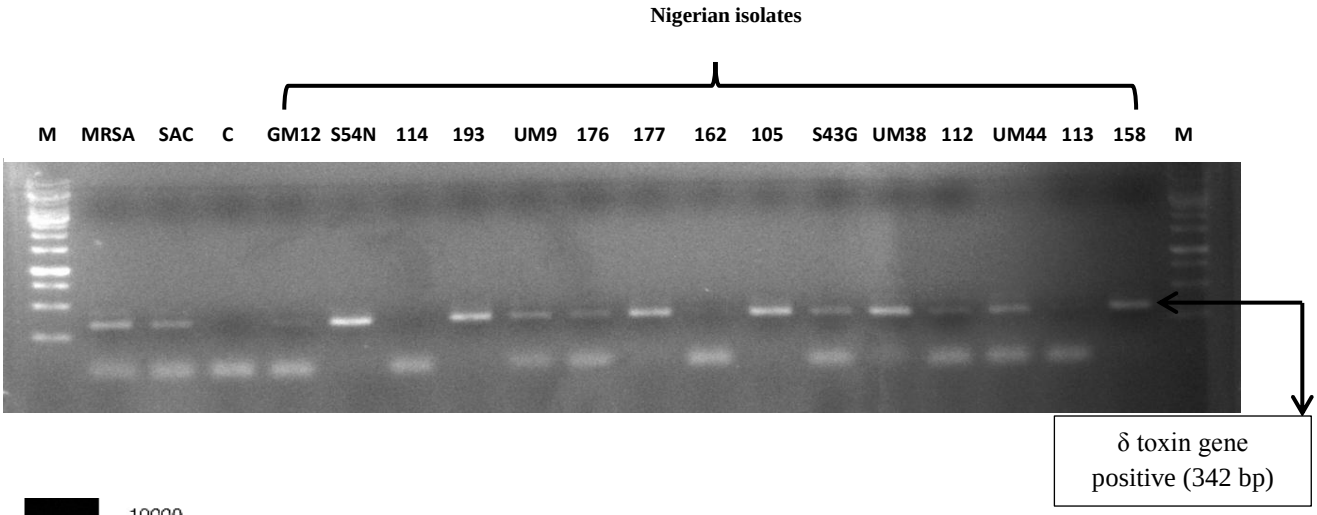


Figure 2.16: Representative gel showing products obtained from the PCR to amplify an 830 bp region of the beta toxin gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

Further to these, all of the UK isolates were seen to bear both the δ toxin and PSMα genes (Appendix D Figures D.15 and D.17 respectively) while only 66.67% (16/11, Nasal, 2, 3) of them bear the γ-haemolysin (*hlγ*) gene (Appendix D Figure D.16). On the other hand, all the Nigerian isolates were seen to bear both the *hlγ* and PSMα genes (Figures 2.18 and 2.19 respectively) while just four (25%) (isolates 114, 162, 113 and 178) of them did not bear the δ toxin gene (Figures 2.17 and D.15 [Appendix D]).



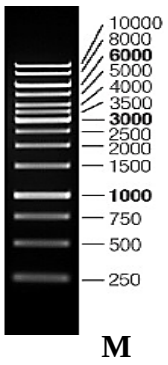
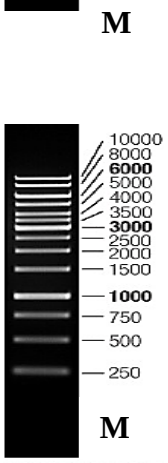


Figure 2.17: Representative gel showing products obtained from the PCR to amplify a 342 bp region of the delta toxin gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).



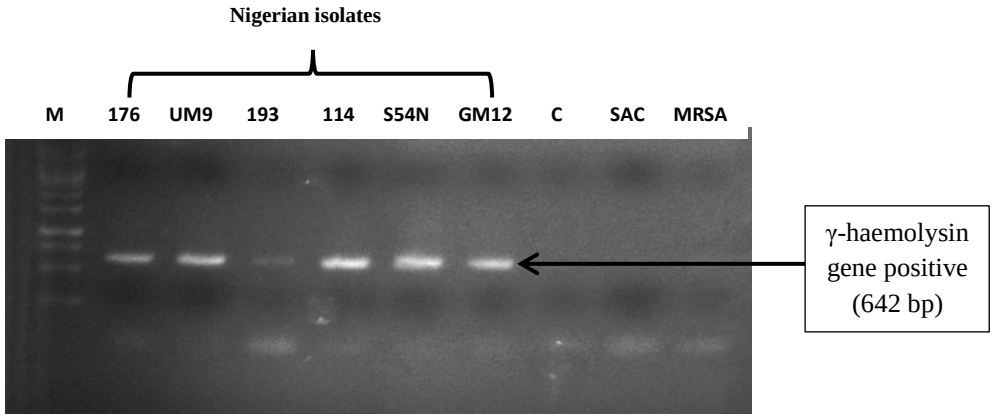


Figure 2.18: Representative gel showing products obtained from the PCR to amplify a 642 bp region of the gamma haemolysin gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

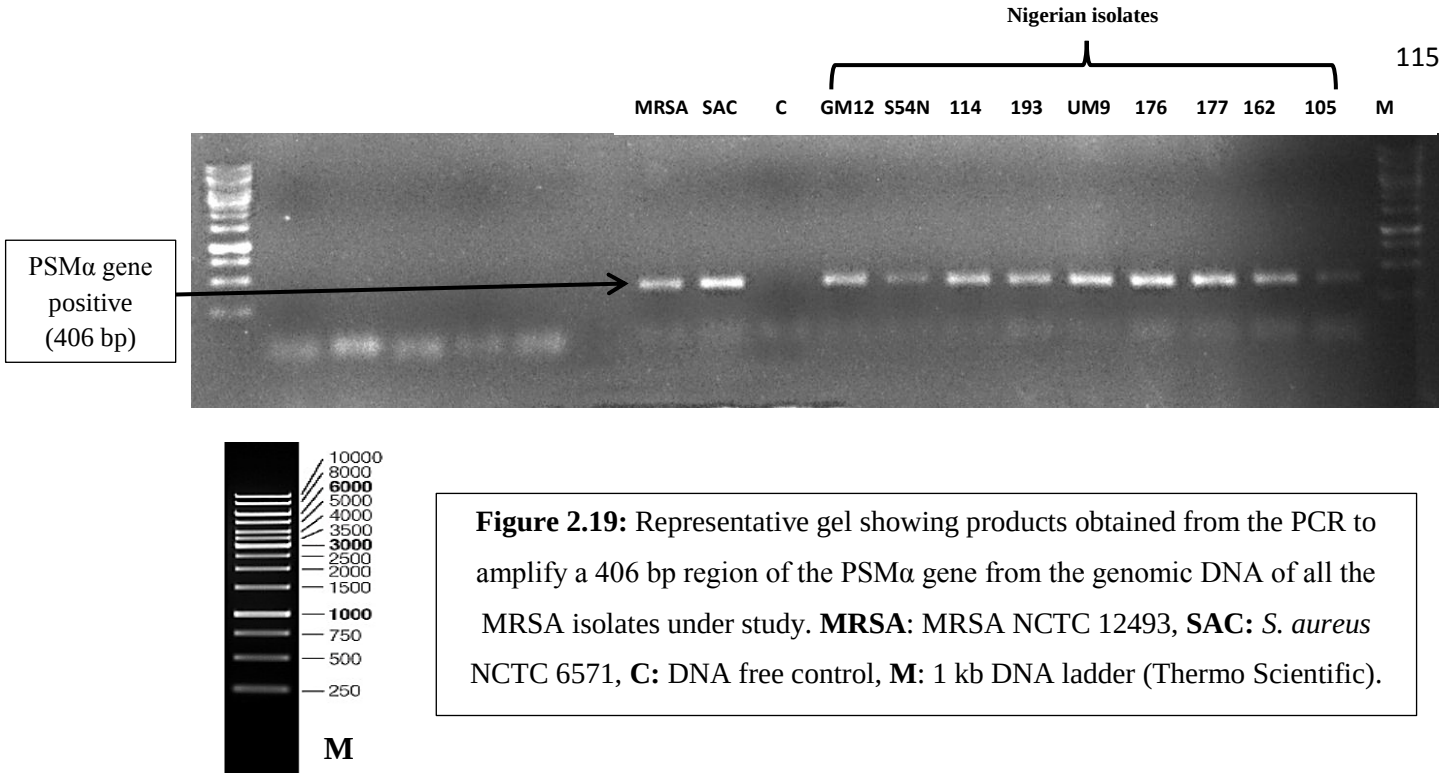


Figure 2.19: Representative gel showing products obtained from the PCR to amplify a 406 bp region of the PSMα gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

Capsular polysaccharide serotype 5 was found to be present in 66.67% of the UK isolates (16/11, Nasal, 2, 3) and in 18.75% of the Nigerian isolates (GM12, S54N, S43G) (Figures 2.20 and D.18 [Appendix D])). On the other hand, the presence of Cap8 was confirmed in 16.17% of the UK isolates (isolate Shin) and 56.25% of the Nigerian isolates (114, UM9, 176, 177, 162, 105, UM38, UM44 and 178) (Figures 2.21 and D.19 {Appendix D]).

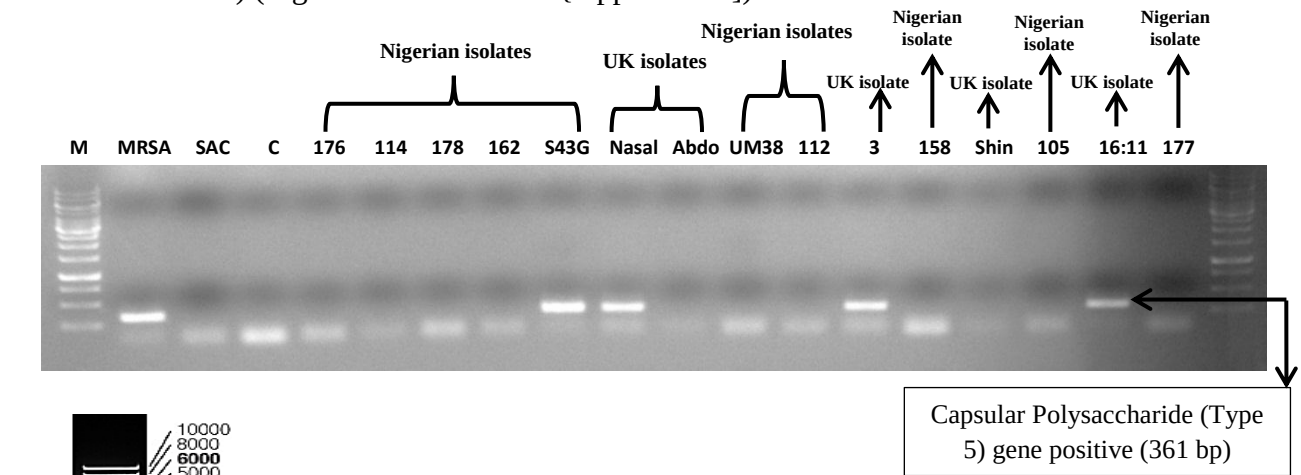
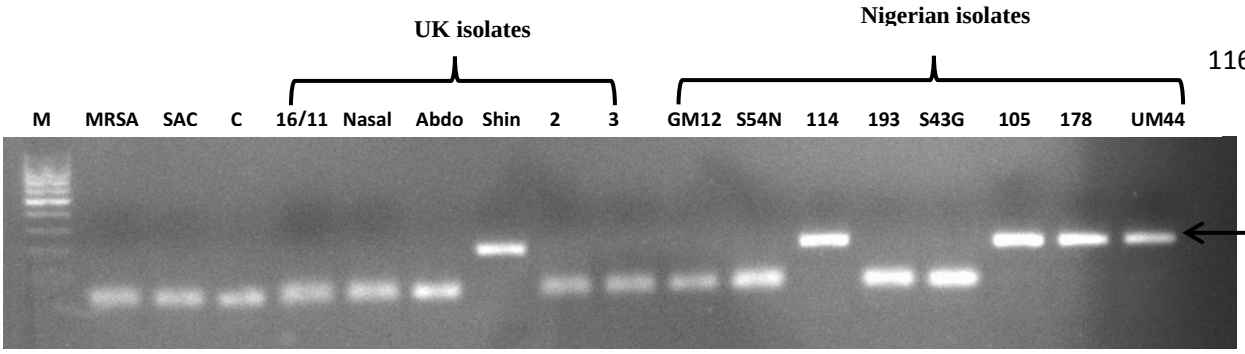
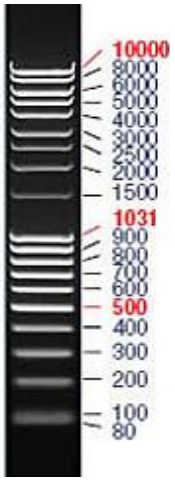


Figure 2.20: Representative gel showing products obtained from the PCR to amplify a 361 bp region of the capsular polysaccharide (type 5) gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).



Capsular Polysaccharide (Type 8) gene positive (173 bp)

Figure 2.21: Representative gel showing products obtained from the PCR to amplify a 173 bp region of the capsular polysaccharide (type 8) gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: MassRuler DNA Ladder (Thermo Scientific).



M

However, none of the MRSA isolates (both UK and Nigerian) were seen to bear the PVL gene (Gels not shown).

A summary of the virulence factors found to be present in the different MRSA isolates (UK and Nigerian) is as seen in Table 2.3.

Table 2.3: Summary of results from the PCR amplification of various virulence factors

	Genes amplified												
Isolates	***spa***	***tst***	**entB**	**entC**	***hla***	***hlb***	***δ toxin***	***hlγ***	**PSMα**	***fnbA***	***fnbB***	**Cap5**	**Cap8**
UK													
16/11	+			+	+	+	+	+	+	+	+	+	
Nasal	+			+	+		+	+	+	+		+	
Abdo	+	+			+		+		+	+			
Shin	+	+			+	+	+		+	+			+
2	+			+	+	+	+	+	+	+		+	
3	+				+		+	+	+	+		+	
Total (%)	**100**	**33.33**	**0**	**50**	**100**	**50**	**100**	**66.67**	**100**	**100**	**16.67**	**66.67**	**16.67**
Nigerian													
GM12	+				+	+	+	+	+	+	+	+	
S54N	+		+		+	+	+	+	+	+	+	+	
114	+					+		+	+	+	+		+
193	+					+	+	+	+	+	+		
UM9	+				+	+	+	+	+	+			+
176	+				+	+	+	+	+	+	+		+
177	+			+	+	+	+	+	+	+	+		+
162	+			+		+		+	+	+	+		+
105	+					+	+	+	+	+	+		+
S43G	+		+		+	+	+	+	+	+	+	+	
UM38	+				+	+	+	+	+	+			+
112	+				+	+	+	+	+	+	+		
UM44	+					+	+	+	+	+	+		+
113	+					+		+	+	+	+		
158	+					+	+	+	+	+	+		
178	+			+		+		+	+	+	+		+
Total (%)	**100**	**0**	**12.5**	**18.75**	**50**	**100**	**75**	**100**	**100**	**100**	**87.5**	**18.75**	**56.25**
***S. aureus* NCTC 6571**	+				+					+			
MRSA NCTC 12493	+				+	+				+		+	

spa**:** *S. aureus* protein A; ***tst*****:** toxic shock syndrome toxin; **entB:** enterotoxin B; **entC:** enterotoxin C; ***hla*****:** alpha toxin/haemolysin; ***hlb***: beta toxin/haemolysin ; ***δ*****-toxin*:*** delta toxin; ***hlγ;*** gamma toxin/haemolysin; **PSMα:** phenol soluble modulin alpha; ***fnbA*****:** fibronectin binding protein A; ***fnbB*****:** fibronectin binding protein B; **Cap5:** capsular polysaccharise type 5; **Cap8:** capsular polysaccharide type 8

2.3.4. Clonal typing

Although the distribution of MRSA clones in Africa is not well described (Abdulgader *et al*., 2015), MRSA isolates of the ST239 lineage have been found to be a cause of infections in Nigeria (Omuse *et al*., 2016). Furthermore, MRSA of the CC22 and CC30 lineages are the major clones that dominate clinical UK populations (Knight *et al*., 2012; Chatterjee and Otto 2013). Therefore, primers (listed in Table E.1 [Appendix E]) were used in PCR to ascertain if the MRSA isolates belong to ST239, CC30 or CC22 lineages. The 120 bp region of DNA, specific to the *sasX* gene of ST239 MRSA strains was not amplified from the genomic DNA of any of the UK isolates. However, the 120 bp region was amplified from the genomes of 11 (68.75%) of the Nigerian MRSA isolates (Figures 2.22 and D.20 {Appendix D]).

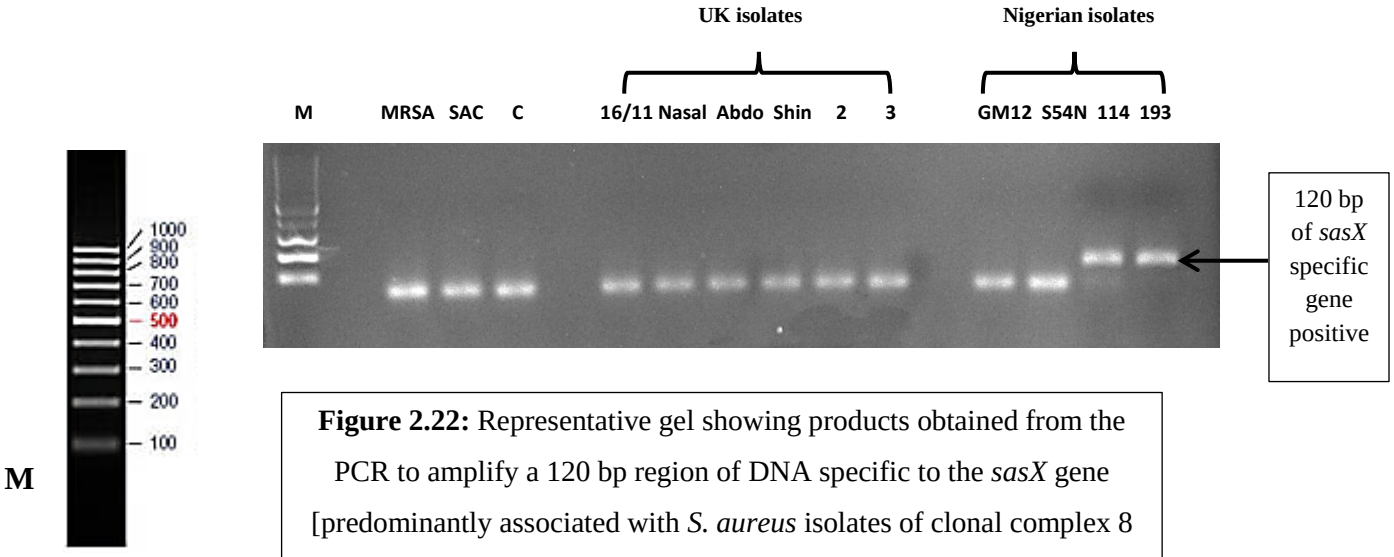
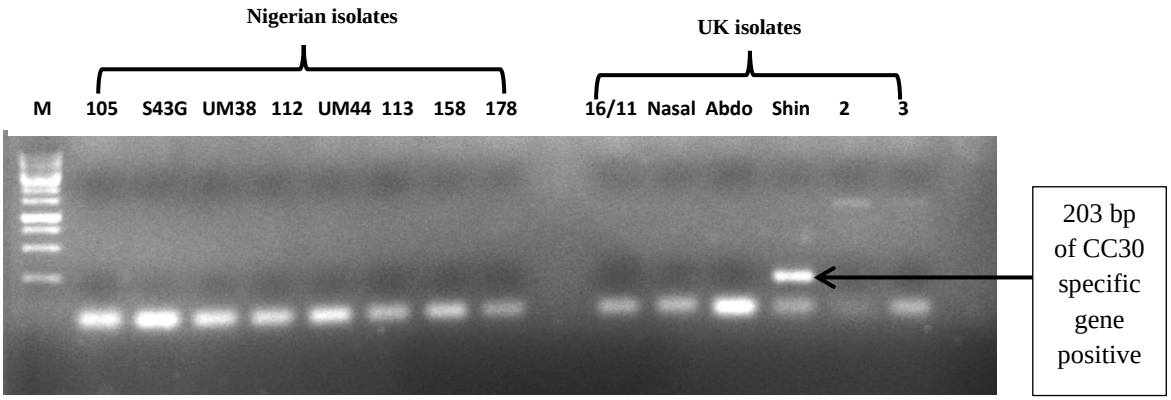


Figure 2.22: Representative gel showing products obtained from the PCR to amplify a 120 bp region of DNA specific to the *sasX* gene [predominantly associated with *S. aureus* isolates of clonal complex 8 (ST239)] from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

Only one of the isolates (UK isolate Shin) was seen to belong to CC30 as it was the only isolate from which a 203 bp region, specific to CC30 MRSA isolates, was amplified (Figure 2.20)



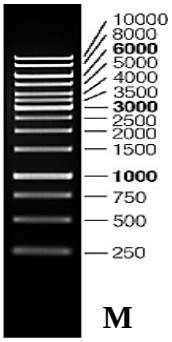


Figure 2.23: Representative gel showing products obtained from the PCR to amplify a 203 bp region of DNA specific to *S. aureus* isolates of clonal complex 30 (CC30) from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).

PCR amplification of DNA specific to MRSA isolates of the CC22 lineage showed that the expected PCR product of ~990 bp was amplified from the genomic DNA of UK isolates Nasal, 2. A smaller DNA fragment was however also amplified from the genomic DNA of isolate Shin using the same primer set.

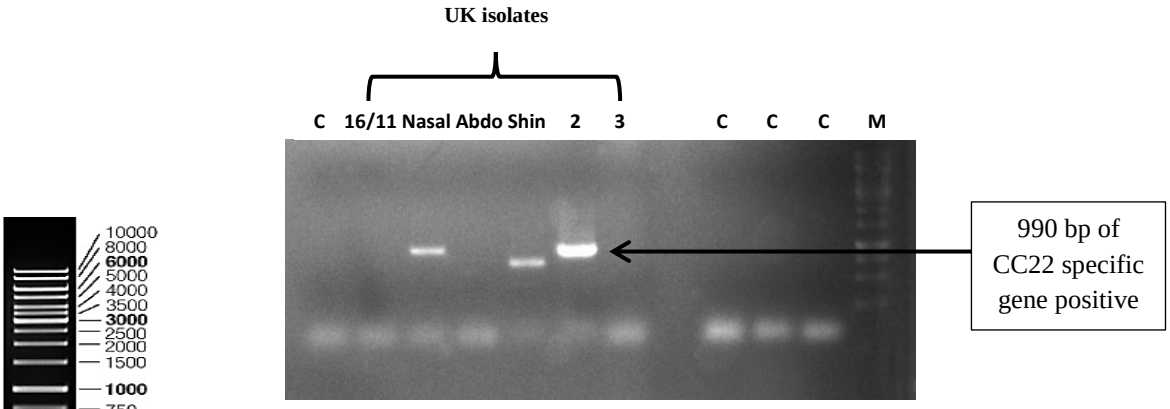


Figure 2.24: Representative gel showing products obtained from the PCR to amplify a 990 bp region of DNA specific to *S. aureus* isolates of clonal complex 22 (CC22) from the genomic DNA of all the MRSA isolates under study. **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).

2.3.5. Antibiotic disc diffusion test

Results obtained from the disc diffusion test (Table 2.4) show that nine (56.25%) of the Nigerian MRSA isolates (114, 193, 176, 162, 112, UM44, 113, 158 and 178) are resistant to > 5 of the 12 antibiotics used in this study (Table 2.4). On the other hand, only one (16.67%) UK MRSA isolate (16/11) was resistant to at least five of the antibiotics (Table 2.4). While only one UK isolate (Shin) was susceptible to all the antibiotics, all (100%) the Nigerian isolates were resistant to at least one of the antibiotics (Table 2.4). No Nigerian isolate was resistant to fusidic acid, but there were two (33.33%) of the UK isolates (16/11 and 3) that were resistant to it. Eleven (68.75%) of the Nigerian MRSA were resistant to clindamycin, but all of the UK isolates were susceptible to it. In addition, all the MRSA isolates (UK and Nigerian) were susceptible to the antibiotics linezolid, tigecycline, rifampin and quinupristin/dalfopristin. Figure 2.25 is a diagrammatic summary of the proportions of the MRSA isolates resistant to the antibiotics under study.

Table 2.4: Antibiotic susceptibility patterns (based on the antibiotic disc diffusion test) of all the MRSA isolates (UK and Nigerian) under study to 12 antibiotics

Isolate	FOX 30 µg	LZD 10 µg	FD 10 µg	TE 30 µg	AK 30 µg	CIP 5 µg	E 15 µg	DA 2 µg	TGC 15 µg	QD 15 µg	RD 5 µg	CN 10 µg	% Resistance
UK													
16/11	R	S	R	R	S	R	R	S	S	S	S	S	**41.67**
Nasal	R	S	S	R	S	R	R	S	S	S	S	S	**33.33**
Abdo	R	S	S	S	S	R	R	S	S	S	S	S	**25.00**
Shin	S	S	S	S	S	S	S	S	S	S	S	S	**0.00**
2	S	S	S	S	S	R	S	S	S	S	S	S	**8.33**
3	R	S	R	S	S	R	S	S	S	S	S	S	**25.00**
Proportion of Resistant isolates (%)	**66.67**	**0.00**	**33.33**	**33.33**	**0.00**	**83.33**	**50.00**	**0.00**	**0.00**	**0.00**	**0.00**	**0.00**	
Nigerian													
GM12	R	S	S	R	S	R	S	S	S	S	S	S	**25.00**
S54N	S	S	S	R	S	R	S	S	S	S	S	S	**16.67**
114	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
193	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
UM9	S	S	S	R	S	S	S	S	S	S	S	S	**8.33**
176	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
177	R	S	S	R	S	R	R	R	S	S	S	R	**50.00**
162	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
105	R	S	S	R	S	R	R	R	S	S	S	S	**41.67**
S43G	S	S	S	R	S	R	S	S	S	S	S	S	**16.67**
UM38	S	S	S	R	S	S	S	S	S	S	S	S	**8.33**
112	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
UM44	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
113	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
158	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
178	R	S	S	R	R	R	R	R	S	S	S	R	**58.33**
Proportion of Resistant isolates (%)	**75.00**	**0.00**	**0.00**	**100.00**	**56.25**	**87.50**	**68.75**	**68.75**	**0.00**	**0.00**	**0.00**	**62.50**	

Cefoxitin (**FOX**), Linezolid (**LZD**), Fusidic acid (**FD**), Tetracycline (**TE**), Clindamycin (**DA**), Ciprofloxacin (**CIP**), Amikacin (**AK**), Erythromycin (**E**), Tigecycline (**TGC**), Quinupristin/Dalfopristin (**QD**), Rifampicin (**RD**) and Gentamicin (**CN**)

[**S:** Susceptible; **R:** Resistant]

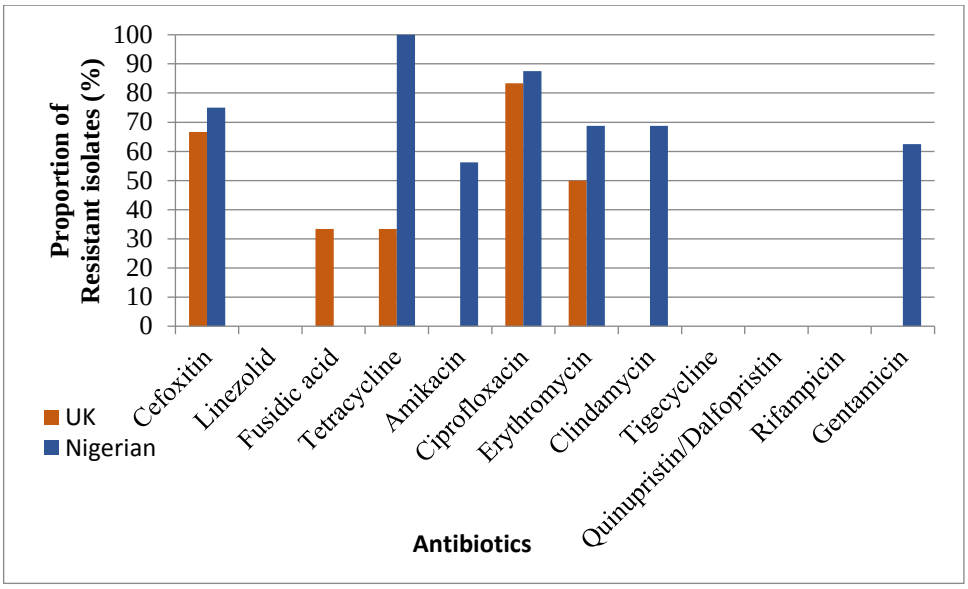


Figure 2.25: Proportions of MRSA isolates resistant to the antibiotics under study (based on the antibiotic disc diffusion test).

Table 2.5: Summary of the characteristics of the MRSA isolates based on results from SCC*mec* typing, clonal typing PCR amplification of virulence factors, and antibiotic disc diffusion test. Isolates of the ST239 lineage and the corresponding virulence genes they bear are in red.

Isolate	SCC*mec* type	Clonal type	% Resistance to 12 antibiotics	Genes amplified												
				Spa	*tst*	ent B	ent C	*hla*	*hlb*	*hlδ*	*hlγ*	PSM*α*	*fnb A*	*fnb B*	Cap 5	Cap 8
UK isolates																
16/11			41.67	+			+	+	+	+	+	+	+	+	+	
Nasal		CC22	33.33	+			+	+		+	+	+	+		+	
Abdo	II		25.00	+	+			+		+		+	+			
Shin	I	CC30	0.00	+	+			+	+	+		+	+			+
2		CC22	8.33	+			+	+	+	+	+	+	+		+	
3			25.00	+				+		+	+	+	+		+	
Nigerian isolates																
114	III	ST239	58.33	+					+		+	+	+	+		+
193	III	ST239	58.33	+					+	+	+	+	+	+		
176	III	ST239	58.33	+				+	+	+	+	+	+	+		+
177	III	ST239	50.00	+			+	+	+	+	+	+	+	+		+
162	III	ST239	58.33	+			+		+		+	+	+	+		+
105	II	ST239	41.67	+					+	+	+	+	+	+		+
112	III	ST239	58.33	+				+	+	+	+	+	+	+		
UM44		ST239	58.33	+					+	+	+	+	+	+		+
113	III	ST239	58.33	+					+		+	+	+	+		
158	III	ST239	58.33	+					+	+	+	+	+	+		
178	III	ST239	58.33	+			+		+		+	+	+	+		+
GM12			25.00	+				+	+	+	+	+	+	+	+	
S54N	V		16.67	+		+		+	+	+	+	+	+	+	+	
UM9			8.33	+				+	+	+	+	+	+			+
S43G	V		16.67	+		+		+	+	+	+	+	+	+	+	
UM38			8.33	+				+	+	+	+	+	+			+

spa*: *S. aureus protein A; ***tst*:** toxic shock syndrome toxin; **entB:** enterotoxin B; **entC:** enterotoxin C; ***hla*:** alpha toxin/haemolysin; ***hlb***: beta toxin/haemolysin ; ***δ*-toxin:** delta toxin; ***hlγ;*** gamma toxin/haemolysin; **PSM*α*:** phenol soluble modulin alpha; ***fnbA*:** fibronectin binding protein A; ***fnbB*:** fibronectin binding protein B; **Cap5:** capsular polysaccharise type 5; **Cap8:** capsular polysaccharide type 8

2.3.6. Minimum inhibitory concentration (MIC) micro-dilution assays

Minimum inhibitory concentration micro-dilution assays were performed to determine the susceptibility of all the MRSA isolates (UK and Nigerian) under study to cefoxitin, tetracycline, vancomycin, ciprofloxacin and gentamicin. The MIC is the lowest concentration of an antimicrobial agent that completely visually inhibits 99% growth of the microorganisms after overnight incubation (Ansari *et al*., 2011; Sultan *et al*., 2015). Therefore, concentrations at which the bacterial inhibition rate (BIR) equals 99% is equivalent to the MIC for that antibiotic (BIR- 99 = MIC).

2.3.6.1. Cefoxitin

According to Version 7.1 of the EUCAST guidelines, an MIC breakpoint of > 4µg/ml means that *S. aureus* is resistant to cefoxitin. Results from this study showed that all the MRSA isolates (UK and Nigerian) were resistant to cefoxitin, i.e. they have MIC values > 4µg/ml. This is because cefoxitin caused inhibition of up to 99% for all the isolates at concentrations $\geq$ 6µg/ml (Tables 2.6 A and B). Based on the results, all the UK isolates (100%) and 14 of the Nigerian isolates (87.5%) have cefoxitin MICs to be > 16µg/ml (Tables 2.6 A and B). The MICs of cefoxitin for Nigerian isolates GM12 and UM9 were 6µg/ml and 8µg/ml respectively (Table 2.6B). Figure 2.26 is a diagrammatic summary of the distribution of the Nigerian and UK isolates based on the inhibitory effect of cefoxitin. In summary, the results showed that the MIC of cefoxitin was > 24µg/ml for 100% (all 6) of the UK isolates (Table 2.6A) compared to 87.5% (14) of the Nigerian isolates (Table 2.6B).

Table 2.6A: Bacterial inhibition rates (BIRs) of different concentrations of cefoxitin on UK MRSA isolates after 24-hour incubation under aerobic conditions. Results are the mean of three independent experiments.

Cefoxitin (µg/ml)	UK MRSA Isolates					
	16::11	Nasal	Abdo	Shin	2	3
0	0	0	0	0	0	0
6	87.0	89.1	93.7	86.3	88.1	84.1
8	88.7	90.5	94.4	92.7	89.0	86.8
12	91.2	91.3	96.4	94.0	90.8	88.9
16	93.8	92.5	97.2	95.3	92.8	90.0
24	95.0	93.3	97.6	96.8	95.3	92.0

Table 2.6B: Bacterial inhibition rates (BIRs) of different concentrations of cefoxitin on Nigerian MRSA isolates after 24-hour incubation under aerobic conditions. 99% BIRs are highlighted in yellow. Results are the mean of three independent experiments.

Cefoxitin (µg/ml)	Nigerian MRSA Isolates															
	GM12	S54N	105	177	114	178	113	S43G	158	UM44	UM38	162	176	193	112	UM9
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	98.6	88.0	90.0	93.7	88.5	89.5	88.9	82.0	89.2	82.8	91.0	88.7	84.1	96.1	93.5	97.5
8	99.2	91.0	92.5	94.3	89.9	90.5	91.8	85.8	89.8	87.9	92.4	89.3	85.7	96.6	95.7	98.6
12	99.5	91.4	93.1	95.0	90.8	91.8	92.9	88.1	91.6	88.8	93.0	90.4	86.7	97.2	96.5	98.9
16	99.6	93.3	95.0	95.4	92.6	92.3	93.9	90.9	92.1	92.5	94.8	92.5	88.0	97.4	97.4	99.3
24	99.8	95.2	96.3	96.1	95.3	93.8	95.3	95.5	92.5	93.9	95.9	93.1	90.3	98.1	97.9	99.5

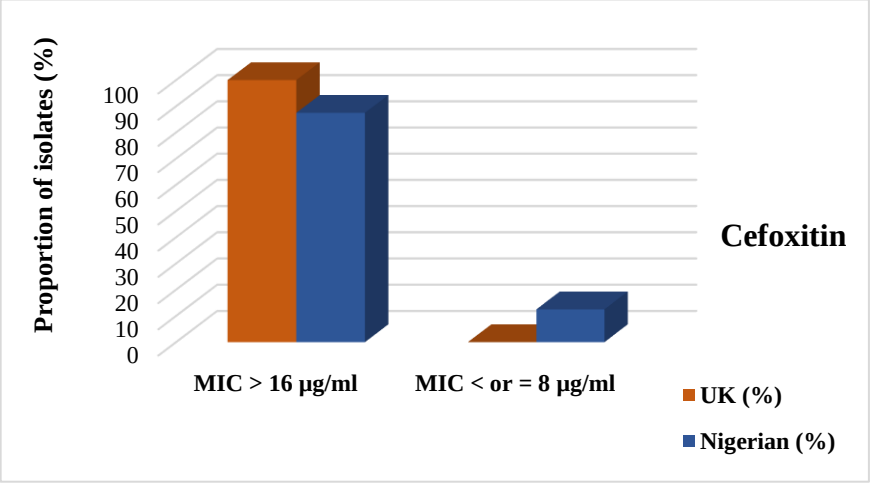


Figure 2.26: Distribution of UK and Nigerian MRSA isolates based on cefoxitin MIC

2.3.6.2. Tetracycline

An MIC breakpoint of > 2µg/ml means that staphylococci are resistant to tetracycline according to EUCAST version 7.1. Results from this study showed that while only 14 (87.5%) of the Nigerian isolates were also resistant to tetracycline, all (100%) of the UK isolates were resistant to tetracycline because tetracycline inhibited up to 99% growth of the isolates at concentrations > 2µg/ml (Tables 2.7 A and B). Furthermore, the results showed that the MIC of tetracycline against nine (56.25%) of the Nigerian isolates are $\geq$ 6µg/ml (Table 2.7B) while only two (33.33%, i.e. isolates 16/11 and Abdo) of the UK isolates were seen to have MICs $\geq$ 6µg/ml (Table 2.7A). Only Nigerian isolates 177, 178 and 113 had tetracycline MICs > 6µg/ml (Table 2.7B). Figure 2.27 is a diagrammatic summary of the distribution of the Nigerian and UK MRSA based on the inhibitory effect of tetracycline.

Table 2.7A: Bacterial inhibition rates (BIRs) of different concentrations of tetracycline on UK MRSA isolates after 24-hour incubation under aerobic conditions. 99% BIRs are highlighted in yellow. Results are the mean of three independent experiments

Tetracycline (µg/ml)	UK MRSA Isolates					
	16::11	Nasal	Abdo	Shin	2	3
0	0	0	0	0	0	0
1	91.1	91.9	86.4	89.4	84.6	86.9
1.5	92.4	95.1	93.0	94.3	92.2	93.6
2	93.6	97.6	94.7	97.9	97.1	96.9
3	95.4	98.2	96.5	99.6	97.8	97.6
4	97.0	99.0	97.9	99.9	99.0	98.7
6	99.3	99.9	99.1	100.0	99.8	99.4

Table 2.7B: Bacterial inhibition rates (BIRs) of different concentrations of tetracycline on Nigerian MRSA isolates after 24-hour incubation under aerobic conditions. 99% BIRs are highlighted in yellow. Results are the mean of three independent experiments

Tetracycline (µg/ml)	Nigerian MRSA Isolates															
	GM12	S54N	105	177	114	178	113	S43G	158	UM44	UM38	162	176	193	112	UM9
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
1	94.5	92.7	97.6	92.9	89.6	93.4	89.7	92.6	94.0	90.8	93.8	88.8	88.2	97.1	98.3	99.4
1.5	94.9	93.3	98.0	95.3	91.4	94.0	91.8	94.0	95.5	92.1	95.1	93.7	90.0	97.6	99.1	99.6
2	96.6	94.7	98.1	96.0	92.1	95.2	93.2	95.0	96.9	93.5	96.5	95.7	94.4	97.7	99.2	99.8
3	98.2	95.4	98.6	97.0	94.5	96.1	95.5	96.0	97.4	95.8	97.8	97.3	96.0	98.2	99.3	99.8
4	98.7	97.8	98.9	97.1	97.3	97.2	96.8	98.1	98.5	96.6	98.2	98.1	98.6	99.0	99.4	99.9
6	99.8	99.4	99.8	97.6	99.6	97.8	98.6	99.6	99.8	99.1	99.7	99.2	99.8	99.8	99.7	99.9

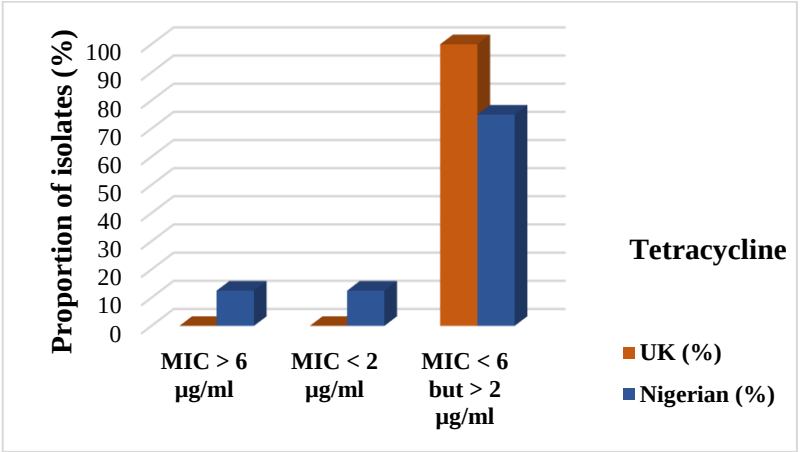


Figure 2.27: Distribution of UK and Nigerian MRSA isolates based on tetracycline MIC

2.3.6.3. Vancomycin

An MIC breakpoint of ≤ 2µg/ml means that *S. aureus* is susceptible to vancomycin according to EUCAST. Results from this study showed that all the MRSA isolates (100% UK and 100% Nigerian) were susceptible to vancomycin as it inhibited growth of up to 99% at concentrations < 2µg/ml (Table 2.8 A and B). A comparison of the UK and Nigerian vancomycin MICs shows that all (100%) of the UK isolates have vancomycin MICs of < 0.75µg/ml while only 10 (62.5%) of the Nigerian isolates have MICs < 0.75µg/ml (Table 2.8 A and B). Furthermore, six (37.5%) of the Nigerian isolates had MICs > 0.75µg/ml but < 2µg/ml (Table 2.8B). Among all the isolates (both UK and Nigerian), Nigerian isolates 177 and 178 had the highest vancomycin MICs at 1.5µg/ml (Table 2.8B). Figure 2.28 is a diagrammatic summary of the distribution of the Nigerian and UK MRSA based on the inhibitory effect of vancomycin.

Table 2.8A: Bacterial inhibition rates of different concentrations of vancomycin on UK MRSA isolates after 24-hour incubation under aerobic conditions. BIRs corresponding to the MICs within the studied concentration range are highlighted in yellow. Results are the mean of three independent experiments

Vancomycin (µg/ml)	UK MRSA Isolates					
	16::11	Nasal	Abdo	Shin	2	3
0	0	0	0	0	0	0
0.75	99.2	99.6	99.5	99.7	99.5	99.1
1	99.4	99.7	99.6	99.8	99.6	99.2
1.5	99.4	99.8	99.7	99.8	99.6	99.2
2	99.6	99.9	99.8	99.9	99.8	99.3

Table 2.8B: Bacterial inhibition rates (BIRs) of different concentrations of vancomycin on Nigerian MRSA isolates after 24-hour incubation under aerobic conditions. BIRs corresponding to the MICs within the studied concentration range are highlighted in yellow. Results are the mean of three independent experiments.

Vancomycin (µg/ml)	Nigerian MRSA Isolates															
	GM12	S54N	105	177	114	178	113	S43G	158	UM44	UM38	162	176	193	112	UM9
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
0.75	99.3	98.2	98.9	97.7	99.1	98.0	98.3	99.3	99.4	99.0	99.6	98.1	98.4	99.6	99.8	99.8
1	99.4	98.7	99.3	98.2	99.2	98.4	98.8	99.4	99.6	99.3	99.7	98.6	99.0	99.6	99.8	99.9
1.5	99.7	99.1	99.5	98.7	99.4	98.8	99.2	99.7	99.7	99.4	99.7	98.9	99.3	99.8	99.8	99.9
2	99.8	99.3	99.7	99.0	99.6	99.1	99.3	99.7	99.8	99.5	99.8	99.2	99.5	99.8	99.9	99.9

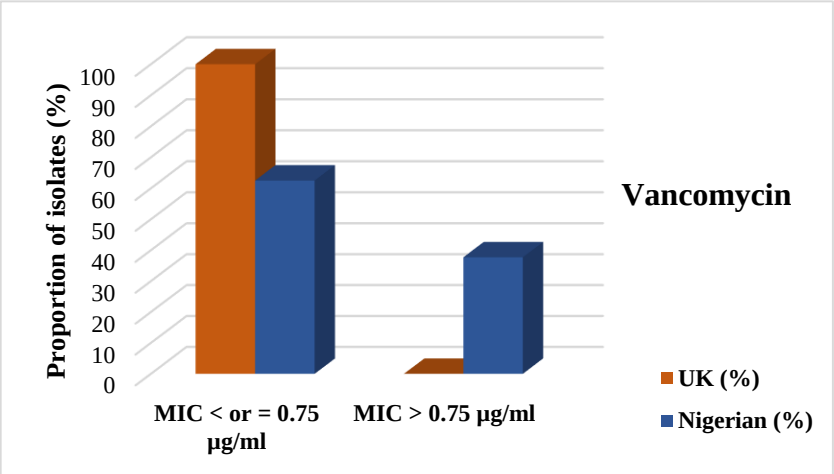


Figure 2.28: Distribution of UK and Nigerian MRSA isolates based on Vancomycin MIC

2.3.6.4. Ciprofloxacin

S. aureus isolates with MICs > 1µg/ml are resistant to ciprofloxacin according to EUCAST guidelines. Ciprofloxacin caused at least 99% inhibition of all (100%) the Nigerian isolates at concentrations ≤ 24µg/ml (Table 2.9B). On the other hand, only 83.33% of the UK isolates have MIC values ≤ 24µg/ml (Table 2.9A). Only one (16.67%) of the UK isolates (isolate Abdo) was seen to have an MIC value > 24µg/ml (Table 2.9A). Furthermore, one (16.67%) UK isolate (isolate Shin) and one (6.25%; Table 2.9A) Nigerian isolate (UM9; Table 2.9B) and were seen to have ciprofloxacin MICs < 8µg/ml, while all the other five UK (83.33%) and 15 Nigerian (93.75%) isolates have MICs > 8µg/ml (Table 2.9 A and B). The UK isolates Abdo had the highest ciprofloxacin MIC of all the isolates at 32µg/ml (Table 2.9B). The distribution of the Nigerian and UK MRSA isolates based on the inhibitory effect of ciprofloxacin is summarised in Figure 2.29.

Table 2.9A: Bacterial inhibition rates of different concentrations of ciprofloxacin on UK MRSA isolates after 24-hour incubation under aerobic conditions. BIRs corresponding to the MICs within the studied concentration range are highlighted in yellow. Results are the mean of three independent experiments

Cipro (µg/ml)	UK MRSA Isolates					
	16::11	Nasal	Abdo	Shin	2	3
0	0	0	0	0	0	0
8	78.8	88.5	36.4	100.0	78.6	81.0
12	81.1	90.0	63.0	100.0	80.5	85.7
16	89.9	93.1	80.4	100.0	88.7	89.3
24	99.2	98.9	97.5	100.0	99.6	98.6
32	100.0	100.0	100.0	100.0	100.0	100.0

Table 2.9B: Bacterial inhibition rates (BIRs) of different concentrations of ciprofloxacin on Nigerian MRSA isolates after 24-hour incubation under aerobic conditions. BIRs corresponding to the MICs within the studied concentration range are highlighted in yellow. Results are the mean of three independent experiments

Cipro (µg/ml)	Nigerian MRSA Isolates															
	GM12	S54N	105	177	114	178	113	S43G	158	UM44	UM38	162	176	193	112	UM9
0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
8	54.6	46.8	90.2	96.2	68.6	89.8	84.7	50.6	93.5	82.2	100.0	86.0	85.2	98.5	98.4	99.9
12	71.9	68.6	90.9	98.0	80.4	91.6	86.9	72.8	95.2	96.1	100.0	89.1	95.9	99.4	99.0	99.9
16	96.4	95.4	95.7	98.7	88.3	95.9	91.5	81.4	96.2	98.5	100.0	95.8	99.7	99.8	99.5	100.0
24	100.0	100.0	100.0	100.0	100.0	99.8	100.0	100.0	99.8	100.0	100.0	100.0	100.0	100.0	100.0	100.0
32	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

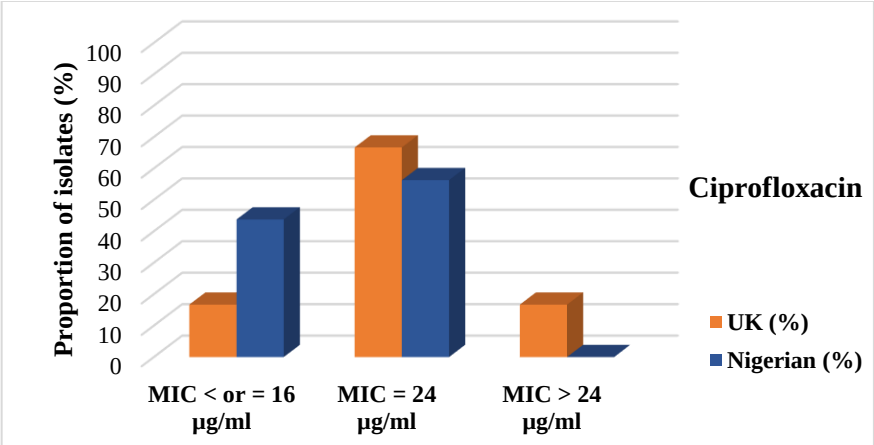


Figure 2.29: Distribution of UK and Nigerian MRSA isolates based on ciprofloxacin MIC

MIC breakpoint values > 1µg/ml means *S. aureus* is resistant to gentamicin according to EUCAST. Results obtained from this study showed that all (100%) of the UK isolates were seen to have MIC values < 6µg/ml because at concentration 6µg/ml, they all have BIRs > 99% (Table 2.10A). However, only six (37.5%) of the Nigerian isolates have MIC values < 6µg/ml (Table 2.10B). Only Nigerian isolate 193 (6.25%) was seen to have an MIC of 32 µg/ml (Table 2.10B) while nine (56.25%) Nigerian isolates had MIC values > 32 µg/ml as gentamicin had not been able to bring about up to 99% inhibition at 32 µg/ml (Table 2.10B). The distribution of the MRSA isolates based on the inhibitory effect of gentamicin is summarised in Figure 2.30. In summary more than half (62.5%) of the Nigerian isolates have MICs > 6µg/ml while all the UK isolates (100%) have MICs < 6µg/ml.

Table 2.10A: Bacterial inhibition rates of different concentrations of gentamicin on UK MRSA isolates after 24-hour incubation under aerobic conditions. BIRs corresponding to the MICs within the studied concentration range are highlighted in yellow. Results are the mean of three independent experiments

Gentamicin (µg/ml)	UK MRSA Isolates					
	16::11	Nasal	Abdo	Shin	2	3
0	0	0	0	0	0	0
6	100.0	100.0	100.0	100.0	100.0	100.0
8	100.0	100.0	100.0	100.0	100.0	100.0
12	100.0	100.0	100.0	100.0	100.0	100.0
16	100.0	100.0	100.0	100.0	100.0	100.0
24	100.0	100.0	100.0	100.0	100.0	100.0
32	100.0	100.0	100.0	100.0	100.0	100.0

Table 2.10B: Bacterial inhibition rates (BIRs) of different concentrations of gentamicin on Nigerian MRSA isolates after 24-hour incubation under aerobic conditions. BIRs corresponding to the MICs within the studied concentration range are highlighted in yellow. Results are the mean of three independent experiments

Gentamicin (µg/ml)	Nigerian MRSA Isolates															
	GM12	S54N	105	177	114	178	113	S43G	158	UM44	UM38	162	176	193	112	UM9
0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
6	100.0	100.0	100.0	59.7	58.3	54.2	78.7	100.0	90.2	84.2	100.0	46.1	79.5	95.6	92.6	100.0
8	100.0	100.0	100.0	57.3	50.9	60.8	80.3	100.0	91.6	80.6	100.0	63.0	83.8	96.1	93.3	100.0
12	100.0	100.0	100.0	61.3	61.8	64.8	81.3	100.0	92.7	82.1	100.0	72.9	86.0	96.6	94.5	100.0
16	100.0	100.0	100.0	69.2	63.5	67.5	82.3	100.0	93.4	85.0	100.0	77.8	89.3	97.0	95.1	100.0
24	100.0	100.0	100.0	73.9	76.0	75.3	85.9	100.0	94.3	91.1	100.0	85.5	90.9	97.9	96.1	100.0
32	100.0	100.0	100.0	76.1	82.7	79.1	90.0	100.0	96.7	95.0	100.0	92.5	95.2	98.5	96.6	100.0

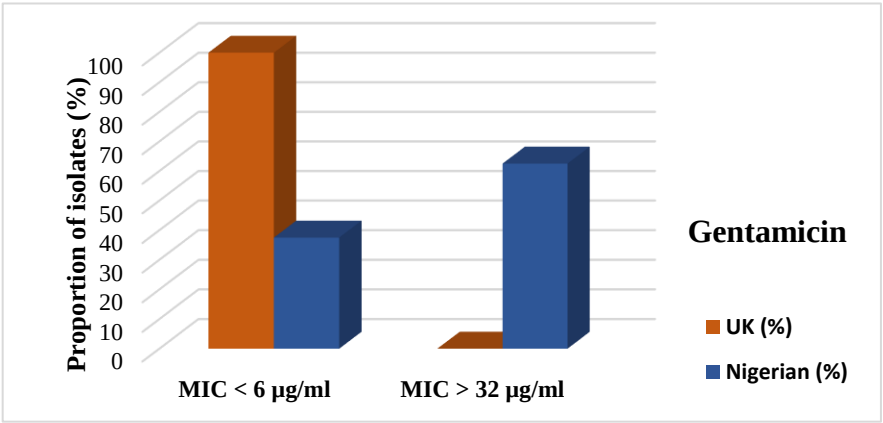


Figure 2.30: Distribution of UK and Nigerian MRSA isolates based on gentamicin MIC

2.4. Discussion

Results obtained from this study showed that a greater percentage of Nigerian MRSA isolates were resistant to more than half of the antibiotics used in the disc susceptibility test compared to their UK counterparts. A greater proportion of the Nigerian MRSA were resistant to seven of the 12 studied antibiotics (cefoxitin-75%, tetracycline-100%, amikacin-56.25%, ciprofloxacin-87.50%, erythromycin-68.75%, clindamycin-68.75% and gentamicin-62.50%) compared to their UK counterparts (cefoxitin-66.67%, tetracycline-33.33%, amikacin-0%, ciprofloxacin-83.33%, erythromycin-50%, clindamycin-0% and gentamicin-0%) (Table 2.5). In addition, nine (56.25%) of the Nigerian MRSA were resistant to at least six of the 12 antibiotics that were studied using the disc susceptibility test. Contrarily, none of the UK isolates were resistant to $\geq$ 5 of the antibiotics under study.

Minimum inhibitory concentration micro-dilution assays were further used to investigate the inhibitory effects of five antibiotics (cefoxitin, tetracycline, vancomycin, ciprofloxacin and gentamicin on all the MRSA isolates (UK and Nigerian) in the course of this study. Similar to results that were obtained from the disc susceptibility test, antimicrobial assays showed that a greater percentage of Nigerian MRSA isolates exhibited a higher level of resistance to more than half of the antibiotics used in the MIC assays compared to their UK counterparts. As seen in section 2.3.6., the Nigerian isolates were more resistant to three [60%] (tetracycline – 2.3.6.2, vancomycin – 2.3.6.3 and gentamicin – 2.3.6.5) of the five antibiotics studied.

Several factors, prevalent in Nigeria and other developing countries, could explain why a higher proportion of the Nigerian MRSA isolates have higher antibiotic MICs than the UK MRSA. One of such factors is the unregulated access to antibiotics. In contrast to northern European and North American countries where antibiotics for ambulatory use are largely restricted to prescription-only, previous studies have shown that non-prescription access to antibiotics in community settings is

common in eastern and southern Europe, Africa, South America and Asia (Morgan *et al*., 2011; Chang *et al*., 2017). Like other low- and middle-income countries, implementation of regulations governing the distribution, sales and use of antibiotics and other prescription medicines in Nigeria is impeded by the government's lack of enforcement staff, budget, and an efficient regulatory and judicial framework (Sanya *et al*., 2013; Akinyadenu and Akinyadenu, 2014). This allows for illicit sale of restricted medicines, often without prescription and by unqualified staff (Akinyadenu and Akinyadenu, 2014). Research has found that the use of antibiotics without prescription is motivated by a complex set of factors which include unchecked sales, time constraints, cost, accessibility, shortage of doctors, influence of friends and family, education level, consumer attitudes and media campaigns (Akinyadenu and Akinyadenu, 2014). Unfortunately, the evidence base regarding the sale of antibiotics without prescription in Nigeria is very small (Akinyadenu and Akinyadenu, 2014). However, studies like those by Esimone *et al*., 2007 and Afolabi *et al*., 2008, have studied utilisation of antimicrobial agents with/without prescription by out-patients in selected pharmacies in South- Eastern Nigeria and assessed the factors influencing self-medication in an adult Nigerian population.

Another factor that could have played a role in the outcome of this study, i.e. a higher proportion of the Nigerian MRSA isolates having higher antibiotic MICs than the UK MRSA, is inappropriate antimicrobial prescriptions (wrong drug, wrong doses, or antimicrobial not necessary at all) (Ayukekbong *et al*., 2017). In Nigeria, antibiotics are often prescribed by health practitioners in the absence of laboratory or clinical evidence of a definite or probable bacterial infection (Akinyadenu and Akinyadenu, 2014). Treatment sometimes consists of administering broad-spectrum antibiotics without a definitive diagnosis and indication for antimicrobial treatment (Ayukekbong *et al*., 2017), hence exposing patients to potential complications of antibiotic therapy (Ventola, 2015). Antimicrobials at low dosages (i.e. residual levels, sub-lethal or sub-therapeutic dosages) have been reported to predispose to emergence of resistant pathogens through promotion

of genetic and phenotypic variability in exposed bacteria due to selection bias (Alhaji and Isola, 2018). While patients with resistant infections can turn to more expensive, newer-generation antibiotics in high-income countries, patients in developing countries might be unable to obtain or afford second-line treatments (Laxminarayan *et al*., 2013). This has led to the proliferation of cheaper but substandard antibiotics in many developing countries. Although proliferation of substandard and adulterated pharmaceutical products is a global public health problem, this phenomenon is of greater concern in many developing countries, like Nigeria, who import most drugs to meet her healthcare need under the guise of affordability (Onanuga and Eboh, 2015).

Drugs with too little or no active ingredient can cause patient death and/or lead to the development of drug resistance (Joda *et al*., 2018). Resistance at the population level renders legitimate drugs and even entire classes of drugs less effective, even for patients who did not previously take poor-quality drugs (Joda *et al*., 2018). Poor drug procurement and distribution practises, low literacy level, inadequate information on the circulation of substandard products, lack of facilities for effective quality control analyses and ineffectiveness of drug regulatory authorities, are some of the factors responsible for the high prevalence of substandard drug products in Nigeria (Onanuga and Eboh, 2015).

Many drivers of antibiotic consumption are based in human medicine, but antibiotic use in veterinary medicine and for growth promotion and disease prevention in agriculture, aquaculture, and horticulture is also a major contributing factor (Laxminarayan *et al*., 2013). Antimicrobials are used to prevent (prophylaxis in high risk animals) and treat diseases in animals, as well as used as growth promoters in animal breeding (Ayukekbong *et al*., 2017). Additionally, they are used as additives in plant agriculture (fruits, vegetables, and orchid, etc.), especially in the spraying of fruit trees for disease prophylaxis and the application of antibiotic-containing manure on farmland and in industrial processes (Ayukekbong *et al*., 2017). Inappropriate use of antimicrobials in the livestock sector contributes to development of antimicrobial resistance that can be transmitted

between animals, humans and the environment (Alhaji and Isola, 2018). A strong association between agricultural use of antimicrobials and the development of resistance has been suggested, and it has been shown that the bulk of antimicrobials used worldwide are not consumed by humans but rather are given to animals for the purposes of food production (Ayukekbong *et al*., 2017). Most of the antimicrobials used in food animals in Nigeria can be obtained without veterinarian's prescription and laws regulating antimicrobial usage in animals are not always enforced (Alhaji and Isola, 2018).

However, science-based information on antimicrobial usage by livestock farmers in food animals in Nigeria is not readily available (Alhaji and Isola, 2018). Unlike in the UK where there are published and readily available/easily accessible guidelines for appropriate use of antibiotics in the treatment of MRSA infections, e.g. Gemmell *et al*., 2006; Gould *et al*., 2009; Nathwani *et al*., 2010; Brown *et al*., 2018; SCAN, 2018; NICE/PHE, 2018, such guidelines for use in Nigeria are also not readily available/easily accessible. Hence, the lack of a comparative analysis on the use individual antibiotics (used in this study) in the UK and Nigeria in this discussion.

The UK MRSA isolates under study were obtained from the Colchester General Hospital and have been confirmed as the causative agents of diseases (Table 2.1). However, based on the SCC*mec* typing scheme used in this study, the results (Table 2.2) showed that only two of the six UK isolates bear either SCC*mec* types I, II, III or VIII which are often found in HA-MRSA strains. Presence of SCC*mec* types I, II, III or VIII in these two isolates supports the probability of them actually being HA-MRSA strains. All the Nigerian MRSA isolates used in this study were obtained from the medical microbiology laboratories hospitals in North-eastern Nigeria (Okon *et al*., 2014). Demographic information about the individuals, from whom the isolates were obtained, were retrieved from the patients' record files (Okon *et al*., 2014), an indication that they were patients in those hospitals and thus have HA-MRSA risk factors. Furthermore, all of them were confirmed as the causative agents of diseases (Table 2.1). Thus, the presence of SCC*mec* types I, II or III in

10 Nigerian isolates (Table 2.2) further supports the likelihood of those isolates being HA-MRSA strains. It is known that successful transfer of strains from the hospital to the community and vice versa has occurred; this has led to "community-acquired hospital onset" and "hospital-acquired community onset" MRSA infections (Scanvic *et al*., 2001; Klevens *et al*., 2006; Becker *et al*., 2014). Therefore, although all the isolates used in this study are of clinical origin, the presence of the SCC*mec* type V (which is often found in CA-MRSA) in Nigerian isolates S54N and S43G means that it is possible that these strains were isolated from patients with 'community-acquired hospital onset' MRSA infections. The possibility of the un-typeable MRSA isolates (based on the SCC*mec* typing scheme used in this study) being of hospital origin cannot be ruled out. This is because it is possible that the use of different primer combinations, not used in this study, could have led to the definite assignment of the SCC*mec* elements borne by un-typeable MRSA. Furthermore, SCC*mec* typing strategies that rely on sequence determination of internal fragments of recombinase genes (Struelens *et al*., 2009; Mehndiratta and Bhalla, 2012) were not used in this study, and these too could have led to the definite assignment of the SCC*mec* elements borne by un-typeable MRSA. Finally, it is also possible that the un-typeable SCC*mec* elements are novel; complete nucleotide sequencing, which is commonly carried out in the case of novel SCC*mec* elements (Shore *et al*., 2012), would shed more light in that regard.

SCC*mec* types I, II III and VIII, often found in HA-MRSA, carry additional drug-resistance genes (IWG-SCC 2009; Kuo *et al*., 2012; Namvar *et al*., 2015). This phenomenon was observed in this study; the results showed that except UK isolate 'Shin' that was susceptible to all the antibiotics used in the disc diffusion tests, all the isolates (UK and Nigerian) that were found to bear either SCC*mec* type I, II, or III were resistant to at least three of the studied antibiotics (Table 2.5).

Although carriage by HA-MRSA has been described, the PVL gene is carried mainly by CA-MRSA (Hu *et al*., 2015). The absence of this virulence factor in any of the MRSA isolates (UK and Nigerian) increases the probability that all the isolates (UK and Nigerian) are HA-MRSA. All

the isolates under study are seen to bear the spa gene and as expected, because the region of the spa gene amplified is the polymorphic X region [that has a high degree of genetic diversity (Sabat *et al*., 2013)], the sizes of the *spa* DNA amplified from all the isolates were varied (Figure 2.9 and Figure D.8 [Appendix D]).

MRSA strains are classified based on the ST and the SCC*mec* element they bear (Deurenberg *et al*., 2007). Isolates from the same clone have the same SCC*mec* type, restriction-modification system, as well as core variable genes (Knight *et al*., 2012). However, they carry different sets of mobile genetic elements (MGEs), thus different combinations of antibiotic resistances (Knight *et al*., 2012). One of the most successful and persistent clones of MRSA, that rarely cause disease outside the hospital, is the globally dispersed multidrug-resistant multilocus sequence type 239 (ST239) (Baines *et al*., 2015). It is a naturally occurring hybrid that originated from a large recombination event involving descendants of the two major staphylococcal lineages i.e. clonal complexes 8 and 30 (Yamamoto *et al*., 2012; Baines *et al*., 2015). CC239 (i.e. ST239 and its single locus variants ST240 and ST241) harbouring the SCC*mec* type III element have been designated various names in different geographic regions e.g. Hungarian Clone, UK-EMRSA−1,−4,−7,−9, or−11, Irish Phenotype III, Irish AR01,−09,−44, and−23, the Brazilian Clone, Australian Epidemic MRSA−2 and−3 as well as Canadian MRSA−3 or−6 (Monecke *et al*., 2018). CC239-MRSA-III has been reported in many countries including the UK, Ireland, Spain, Turkey, Iran, Saudi Arabia, China, Malaysia and Australia (Monecke *et al*., 2018). MRSA of the ST239 lineage have also been found to be a cause of infections in Nigeria (Abdulgader *et al*., 2015; Omuse *et al*., 2016) and other African countries like Ghana, Kenya, Niger, Senegal, and South Africa (Abdulgader *et al*., 2015). The gene for the *S. aureus* surface protein, *sasX*, is borne on a MGE and has been linked mostly to HA-MRSA strains belonging to sequence type 239 (Otto, 2012). *SasX* is a virulence factor thought to have a key role in nasal colonisation, pathogenesis of lung disease, and abscess formation (Li *et al*., 2012; Monecke *et al*., 2018).

Amplification of a 120 bp region of this *sasX* gene from the genomic DNA of 11 of the 16 Nigerian isolates indicates that those 11 isolates are likely of the ST239 lineage. All the Nigerian MRSA isolates are of hospital origin, nine of the isolates that bore the *sasX* gene were also seen to bear the SCC*mec* type III element (Table 2.4) often found in HA-MRSA. Presence of the *sasX* gene and the SCC*mec* type III elements are features typical of MRSA that belong to the ST239 lineage. Furthermore, the ST239 MRSA lineage is considered a representative multiple drug resistant HA-MRSA circulating worldwide (Yamamoto *et al*., 2012). Li *et al*., 2012 have previously found that MRSA strains that bore the *sasX* gene spread rapidly; thus, presence of the *sasX* gene may account for why the ST239-*sasX* positive MRSA lineage predominated among the MRSA isolates in this study. Typical of MRSA isolates belonging to the ST239 lineage, the Nigerian MRSA isolates found to be of the ST239 lineage in this study were multidrug resistant i.e. they were resistant to at least five (41.67%) of the 12 antibiotics used in the disc susceptibility test (Table 2.5). In terms of virulence factors, all the ST239 isolates in this study were seen to have the *spa*, *fnbA* and *fnbB* genes (that encode surface proteins) as well as the *hlb, hlγ* and PSMα genes (that encode cytotoxins) in common. A summary of the antibiotic resistance profile and the virulence factors borne by all the MRSA isolates identified to be of the ST239 lineage, in this study, are as seen in Table 2.5.

CC22/SCC*mec*IV [UK epidemic MRSA (EMRSA)-15] and CC30/SCC*mec*II [UK EMRSA-16] are the major clones that dominate clinical UK populations (Knight *et al*., 2012; Chatterjee and Otto 2013). The CC22 and CC30 strains constitute more than 60% and 35% (respectively) of MRSA associated infections in the UK (Kamaruzzaman *et al*., 2016). In line with this, results from this study showed that the proportion of the UK MRSA isolates belonging to the CC22 lineage is higher than the proportion that belong to the CC30 lineage; i.e. two (33.33%) UK isolates, isolates ‘Nasal and 2’, belong to the CC22 lineage while only one (16.67%) of the UK MRSA isolates, isolate ‘Shin’, belongs to the CC30 lineage. However, whether isolates ‘Nasal and 2’ bear the

SCC*mec*IV element, typical of the CC22/SCC*mec*IV, could not be ascertained with the SCC*mec* typing scheme used in this study.

PCR results showed that a DNA band smaller than was expected following PCR with primers (1 CC22 and 2 CC22 – Table E.1 [Appendix]) specific to DNA from MRSA of the CC22 lineage was also amplified from the genomic DNA of UK isolate 'Shin' (Figure 2.24). Furthermore, although isolate 'Shin' was seen to belong to the CC30 lineage (Table 2.5) it bore the SCC*mec* type I element rather than the type II SCC*mec* element typical of the CC30/SCC*mec*II clones that are widespread in the UK. These findings point to the possibility of isolate Shin being just a variant of the CC22 lineage or that like MRSA isolates of the ST239 lineage, UK isolate Shin is a hybrid of the CC22 and CC30 lineages. Isolate 'Shin' was the only isolate of all the studied MRSA isolates (both UK and Nigerian) that was susceptible to all the antibiotics used for the disc susceptibility test. A summary of the antibiotic resistance profile and the virulence factors borne by all the MRSA isolates identified to be of the ST22 and ST30 lineages, in this study, are as seen in Table 2.5.

Findings from this study bear several differences and similarities to the study by Okon *et al*., 2014, who have previously studied the Nigerian MRSA isolates used in this study. They reported that all the MRSA isolates were PVL negative, which was also the case in this study as there was no DNA amplified from the genomic DNA of the isolates when the Luk-PV-1 and Luk-PV-2 primers (Table E.1 [Appendix E]), specific to a 433 bp region of the PVL gene, were used in PCR. With regards clonal typing, Okon *et al*., 2014 used a combination of MLST of representative strains from *spa* types, and subsequent clonal complex assignment by applying the definition of six (out of seven) shared alleles using eBURST and the MLST database (http://saureus.mlst.net). This way, they found that the MRSA belonging to the ST241 lineage was predominant among the MRSA isolates. They also found that the MRSA-ST241 from 2007 possessed SCC*mec* type I and were all resistant to erythromycin and gentamicin. On the other hand, the clonal typing scheme used in this study

involved the use of primers (listed in Table E.1 [Appendix E]) in PCR to ascertain if the MRSA isolates belong to ST239, CC30 or CC22 lineages. Amplification of the 120 bp region of DNA, specific to the *sasX* gene of ST239 MRSA from the genomes of 11 (68.75%) of the Nigerian MRSA isolates (Figures 2.22 and D.20 [Appendix D]) led to the belief that these Nigerian isolates are likely of the ST239 lineage. Furthermore, results from this study showed that the 2007 Nigerian isolates found to belong to the MRSA-ST239 lineage (i.e. isolates 114, 193, 176, 177, 162, 112, 113, 158 and 178) possess the SCC*mec* type III element not type I (Table 2.5). These differences in the clonal and SCC*mec* typing results from the Okon *et al*., 2014 study and this study, despite the fact that the same MRSA isolates were studied, can be attributed to the different typing schemes used in both studies. The phenomenon whereby the same isolates which were once reported to bear the SCC*mec* type I element (Okon *et al*., 2014) now bearing the SCC*mec* type III element (this study), is similar to the findings from the study by Lina *et al*., 2006. The aim of the study by Lina *et al*., 2006 was to investigate evolutionary relationships among SCC*mec* elements. They found that sequence type-SCC*mec*I was found in SCC*mec* elements types I and IV, suggesting the evolution of an SCC*mec*I element into an SCC*mec*IV element. While Lina *et al*., 2006 proposed that it may have been the result of a genetic excision, it is also possible that SCC*mec* elements were imported/acquired on multiple, independent occasions (Nubel *et al*., 2008). Moreover, MRSA-ST241 is a single locus variant of MRSA-ST239 (i.e. the result of a mutation in the *yqiL* gene [Monecke *et al*., 2011]) and both belong to the same clonal complex i.e. CC239. Despite the difference in the reported SCC*mec* elements borne by the 2007 isolates i.e. SCC*mec*I as reported by Okon *et al*. 2014 (and associated Okon *et al*., 2009) study and SCC*mec* III in this study, both SCC*mec* types I and III are often found in HA-MRSA and all the Nigerian MRSA isolates used in both studies are of hospital origin. Moreover, like the Okon *et al*., 2014 study, all the 2007 MRSA-ST239 isolates (i.e. isolates 114, 193, 176, 177, 162, 112, 113, 158 and 178) were resistant to both erythromycin and gentamicin (Table 2.4).

In summary, results from the disc susceptibility tests showed that, except for UK isolate Shin, all the MRSA isolates under study (UK and Nigerian) were multi-resistant. However, results from the MIC assays showed that a greater percentage of Nigerian MRSA were resistant to more antibiotics. As expected, it was seen that isolates of the CC22 lineage predominated among the UK MRSA isolates while it was isolates that belonged to the globally dispersed multidrug-resistant ST239 lineage that predominated among the Nigerian MRSA. Although strain to strain variation was observed in the distribution of the characterised virulence factors, clonal distribution, as well as distribution based on geographical isolation of the MRSA was observed. For example, the *hlb*, *hlγ*, PSMα, *fnbA* and *fnbB* genes were found to be present in all the ST239 MRSA. In addition, the *hla* and *hlδ* genes were found in all the UK isolates but in only 50% and 75%, respectively, of the Nigerian isolates. This variation in virulence factor distribution may serve as a basis for any differences that may be observed in the virulence potentials of MRSA from UK and Nigeria.

Chapter Three

MRSA Interaction with Mammalian Cell Lines

3.1. Introduction

Staphylococcus aureus has the ability to invade and persist within non-professional phagocytic cells (NPPCs) e.g. osteoblasts, fibroblasts, kidney cells as well as various epithelial cells (Bur *et al*., 2013; Alva-Murillo *et al*., 2014). This ability enables *S. aureus* to evade the innate immune system of the host and to also survive inside a wide variety of mammalian cells (Alva-Murillo *et al*., 2014). By allowing bacteria to 'hide' inside host cells, bacterial internalisation by host cells might also aid evasion of administered antibiotics (Shwarz-Linek *et al*., 2003) thereby resulting in the relapse of a bacterial infection even after a well-conducted antibiotic treatment regimen. Furthermore, the ability to survive within host cells may be crucial for dissemination and infection of distant anatomic sites (Lacoma *et al*., 2017). *Staphylococcus aureus* internalisation into the host cell is preceded by initial adhesion to the host cell (Bur *et al*., 2013; Alva-Murillo *et al*., 2014).

Like other staphylococci, *S. aureus* expresses an array of surface proteins, including the MSCRAMMs (e.g. Fibrinogen-binding, collagen-binding and Fibronectin-binding proteins) and 'secretable expanded repertoire adhesion molecules' (e.g. the extracellular adherence protein-Eap), which are involved in *S. aureus* adhesion to plasma proteins, extracellular matrix or directly to host cells (Bur *et al*., 2013; Alva-Murillo *et al*., 2014; Josse *et al*., 2017). Of all the MSCRAMMs, the Fibronectin-binding proteins (FnBPs – homologues A and B) have been described as the most important virulence factors for host cell invasion (Loffler *et al*., 2014; Ashraf *et al*., 2017). These FnBPs are the main adhesins that bind Fibronectin (Fn) (Brouilette *at al*., 2003), a component of host ECM. Ability to bind fibronectin, a key dimeric glycoprotein in the extracellular matrix, is a characteristic of *S. aureus* adhesion (Alva-Murillo *et al.,* 2014). *S. aureus* adhesion to host cell involves fibronectin (cellular or soluble) forming a bridge between the FnBPs (A and B) and the fibronectin receptor ($\alpha_5\beta_1$ integrin) on the host cell (Josse *et al*., 2017). Bacterial adherence to the cellular surface then induces changes in the cytoskeleton which subsequently leads to the uptake of the bacteria in phagosomes (Loffler *et al*., 2014). *Staphylococcus aureus*-

containing phagosomes eventually fuse with lysosomes, resulting in phagolysosomes, within which most *S. aureus* are degraded, although a fraction of bacteria can persist (Giese *et al*., 2011).

Though *S. aureus* is able to invade host cells via other mechanisms, this FnBP-Fn-$\alpha_5\beta_1$ interaction/bridge is necessary for efficient *S. aureus* internalisation into NPPCs (Alva-Murillo *et al*., 2014). Presence of the *fnbA* and *fnbB* genes, that encode the FnBPs A and B, respectively, varies across the *S. aureus* population (Peacock *et al*., 2000) and at least one of these genes is found in the vast majority of *S. aureus* strains (Brouilette *et al*., 2003). However, despite being closely related, the FnBPA has a higher affinity for fibronectin than FnBPB; moreover, the presence of FnBPA alone in *S. aureus* is sufficient for adhesion and cell invasion (Josse *et al*., 2017). Following host invasion, *S. aureus* could either induce host cell death or reside intracellularly for extended time periods in the cytoplasm (Krut *et al*., 2003; Strobel *et al*., 2016).

Biofilm infections are important clinically because bacteria in biofilms exhibit recalcitrance to antimicrobial compounds and persistence despite sustained host defences (Speziale *et al*., 2014). The development of a bacterial biofilm is a complex, multifactorial process and can be divided into three phases, which involve specific molecular factors: attachment, accumulation/maturation, and detachment/dispersal (Speziale *et al*., 2014). Initial attachment can occur on inert or biotic surfaces (Speziale *et al*., 2014). Attachment of staphylococci to an abiotic surface, such as the naked plastic or metal surface of an indwelling medical device, is dependent on the physicochemical characteristics of the device and bacterial surface components such as the accumulation-associated protein (Aap) (Conlon *et al*., 2014), autolysins AtlA (Houston *et al*., 2011; Bose *et al*., 2012) and AtlE (Rupp *et al*., 2001) or wall teichoic (WTA) and lipoteichoic acids (LTA) (Gross *et al*., 2001). Primary attachment to a biotic surface in host tissues and synthetic surfaces coated with plasma proteins, such as fibronectin, fibrinogen, and vitronectin, is governed by cell wall-anchored proteins including clumping factors A and B and the fibrinogen/fibronectin-binding proteins FnBPA and FnBPB in *S. aureus* (Speziale *et al*., 2014).

Following attachment to surfaces, bacterial cells undergo phenotypic changes, and the process of biofilm maturation begins (Jiang and Pace 2006). Bacteria start to form microcolonies either by aggregation of already attached cells, clonal growth (cell division) or cell recruitment of planktonic cells or cell flocs from the bulk liquid (Jiang and Pace 2006). An extracellular matrix (ECM) composed of proteins, polysaccharides, and/or extracellular DNA (eDNA) is produced, and a complex, three-dimensional structure is established to form a mature biofilm (Sugimoto *et al*., 2018). Inside a biofilm, microbial cells are embedded in ECM, which binds them to each other and to the substrate (Sugimoto *et al*., 2018). ECM maintains structural integrity of biofilms and protects microbes from environmental stresses, attacks by other organisms, and by chemical agents (e.g., antibiotics) (Sugimoto *et al*., 2018). The polysaccharide intercellular adhesin (PIA), encoded by the *ica*ADBC locus (Limoli *et al*., 2015; Oyama *et al*., 2016), promotes adhesive interactions between bacterial cells during biofilm formation (Kot *et al*., 2018). Although the PIA represents a main mechanism of biofilm formation in *S. aureus*, advancements in the study of biofilms have shown the existence, of alternative forms of biofilms that are PIA-independent (Arciola *et al*., 2015). Furthermore, in addition to disseminating genes amongst different microorganisms and acting as a nutrient store, extracellular DNA (eDNA) also strengthens the biofilm matrix of many microorganisms (Jakubovics *et al*., 2013). It is however now recognised that several staphylococcal surface proteins can also promote the accumulation phase in an *ica*-independent manner (Speziale *et al*., 2014). Therefore, CWA proteins mediate primary attachment and also promote intercellular adhesion and biofilm accumulation and maturation (Speziale *et al*., 2014). The dispersal phase is characterised by disruption of the biofilm structure by enzymatic degradation of matrix components, most notably by proteases, nucleases, and a group of small amphiphilic α-helical peptides, known as phenol-soluble modulins (PSMs) functioning as surfactants (Speziale *et al*., 2014).

Following invasion, *S. aureus* has been shown to actively multiply inside human cells (Kubica *et al*., 2008; Koziel *et al*., 2009; Rollin *et al*., 2017); this intracellular replication is known to precede the onset of host cell death (Flannagan *et al*., 2016). As adherence to host cell membranes initiates the invasion process (Strobel *et al*., 2016), this study aimed to investigate/compare the capacities of UK and Nigerian MRSA to associate with (adhere to and be internalised), invade (be internalised) and survive in mammalian cells (KB epithelial and A549 lung cell lines) in interaction assays (where bacteria will be added to adherent cell cultures to facilitate bacteria-cell contact). Furthermore, because adhesion of bacteria to host surfaces is a key element in the formation of biofilms (Ribet and Cossart, 2015), the biofilm forming ability of the MRSA isolates will also be investigated in this study.

3.2. Materials and Methods

All chemicals were purchased from Sigma, unless otherwise stated.

3.2.1. Cell lines and cell culture

Staphylococcus aureus causes both skin and lower respiratory tract infections. Therefore, in this study, human KB epithelial and A549 lung cell lines (carcinoma cells) were used as *in vitro* models to evaluate interaction of MRSA with human skin and lung tissues. KB epithelial cells were cultured and maintained in Minimum Essential Medium Eagle with L-Glutamine (EMEM) while A549 lung cells were cultured and maintained in Dulbecco's Modified Eagle's Medium F-12 (DMEM). Both EMEM and DMEM (catalogue numbers BE12-611F and BE04-687F/U1 respectively from BioWhittaker™-Lonza) were supplemented with 10% v/v foetal bovine serum and 1% minimum essential amino acids. Cells were passaged into T25 flasks and incubated at 37^{o}C in a CO_2 incubator (95% humidity, 5% CO_2). All passages used in this study were between passage numbers 4 to 10.

3.2.2. Cell passage into a T25 flask

Growth medium was aspirated from confluent flasks. The cells were then washed once with phosphate-buffered saline (PBS) following which 0.5 ml accutase was added into the flask. The flask was rocked for a few seconds and then warmed for 1-3 mins at 37^{o}C. Once the cells were totally detached from the tissue culture flask, 5 ml of growth medium were added. The content of the flask was subsequently transferred into a 15 ml centrifuge tube which was then centrifuged for 4 mins at 1000 rpm (134 x g). The supernatant was discarded, and the pellets were re-suspended in warm growth medium. Finally, the suspended cells were passaged into three other flasks and incubated at 37^{o}C in a CO_2 incubator (95% humidity, 5% CO_2).

3.2.3. Seeding cells in 24 and 96 well plates

Cells were detached and re-suspended as described in section 3.2.2 and seeded in 24 or 96-well plates (as required). After this, plates were incubated at 37ºC in a CO_2 incubator (95% humidity, 5% CO_2).

3.2.4. Interaction assays

Interaction of two infectious doses (IDs) i.e. ~10^6 CFU/well and ~10^7 CFU/well of live MRSA with mammalian cells was evaluated in this study. KB and A549 mammalian cell lines were cultured and grown in 24-well plates in appropriate medium and incubated until 80-100% confluence was attained (there are ~10^5 cells in each 80-100% confluent well of a 24-well plate for both KB and A549 lung cell lines). Growth medium from each confluent well was refreshed thereafter following which live MRSA from a 6-hr broth culture was added to each well at the rate of ~10^6 or ~10^7 CFU per well. This corresponds to multiplicity of infections (MOIs) (number of bacteria per cell at the onset of infection) 10:1 and 100:1 respectively. Plates were then incubated at 37ºC (in humidified 95% air, 5% CO_2) for 40 minutes, following which supernatants were discarded and each well washed with PBS to remove non-adherent bacteria.

To ensure that the mammalian cells used during each experiment were proliferating/viable, control wells, free of bacteria, were set up. Cells from one control well were detached, re-suspended and seeded in two other wells; growth in and attachment of cells to the bottom of the new wells, following incubation at 37ºC in a CO_2 incubator (95% humidity, 5% CO_2), showed that the cells used in each experiment were in good condition. Furthermore, a loopful of each 6-hr broth culture was streaked onto CLED agar plates; growth after overnight incubation of the plate at 37ºC confirmed viability of the bacteria used in each experiment. In addition, to check that MRSA could grow in both EMEM and DMEM, bacteria from 6-hr broth cultures were inoculated at a rate of 10^6 and 10^7 CFU per well and incubated overnight. Inoculum from the EMEM and DMEM cultures

were plated onto CLED plates; growth of bacteria following overnight incubation at 37ºC confirmed that both EMEM and DMEM supported growth of MRSA.

3.2.4.1. Bacterial strains and culture

Only eight Nigerian MRSA isolates (GM12, S54N, 114, 193, UM9, 176, 177, 162) and all six UK MRSA isolates (16/11, Nasal, Abdo, Shin, 2, 3) were used for this study. The eight Nigerian isolates were selected based on their virulence factor profiles i.e. the virulence factors checked for in Chapter two. Isolate pairs S54N/S43G, UM9/UM38, 193/158, 162/178 have identical virulence factor profiles and so isolates S54N, UM9, 193 and 162, respectively were selected. The only difference between the virulence profiles of isolates 114 and 105 is that the delta-toxin was absent in isolate 114 and so isolate 114 was selected instead of 105. Isolates GM12 and 176 were both selected as the only difference between them was that isolate GM12 bears the gene for capsular polysaccharide type 8 while isolate 176 bears the gene for capsular polysaccharide type 5. Although isolate 177 had a similar virulence factor profile to isolate 176, it was also selected because it bore the enterotoxin B gene. These isolates were grown and maintained as described in section 2.2.1 (Chapter Two). Optical densities of bacterial broth cultures were measured in a spectrophotometer as also previously described in section 2.2.1 (Chapter Two) i.e. OD_{595} 0.22 = ~10^8 CFU/ml.

3.2.4.2. Association assays

Association assays were done to determine the proportion of MRSA which associate (i.e. adherent and internalised) with the mammalian cells following addition of bacteria. Live MRSA were added to KB skin epithelial and A549 lung cell lines which were cultured and grown as described in section 3.2.4. After removal of non-adherent bacteria, 150 µl of CelLytic M were added to each well to lyse the cells. The number of viable bacteria in each well (which reflect the number of adherent and internalised bacteria) was determined by plating 20 µl (in duplicate) of the content

of each well onto CLED agar plates. The number of colonies on agar was counted on a colony counter (Gallenkamp) after overnight incubation at 37^{o}C.

The proportion (%) of MRSA that associated with the cell lines were calculated as follows:

$$\frac{\text{number of bacteria recovered (CFU)}}{\text{number of bacteria inoculated at the start of the experiment}} \times 100$$

(Alsam *et al*., 2006; Yousuf *et al*., 2013).

3.2.4.3. Invasion assays

To determine the proportion of MRSA that are internalised following addition of bacteria to the study mammalian cell lines, invasion assays were performed. KB skin epithelial and A549 lung cell lines were cultured, grown and bacteria added as described in section 3.2.4. Following removal of non-adherent bacteria, Vancomycin was added to each well at a final concentration of 100 µg/ml and incubated at 37°C (in humidified 95% air, 5% CO_2) for 45 mins to kill extracellular bacteria. Supernatant from each well was then discarded and the wells washed again with PBS. Afterwards, 150 µl of CelLytic M were added to each well to lyse the cells. 20 µl of lysed solution were cultured onto CLED agar plates and incubated overnight at 37°C under aerobic conditions. As done in association assays, the number of viable bacteria in each well was determined by plating 20 µl (in duplicate) of the content of each well onto CLED agar. The number of colonies on agar was counted on a colony counter (Gallenkamp) after overnight incubation at 37^{o}C.

Preliminary experiments were carried out to determine if 100 µg/ml of Vancomycin would effectively kill extracellular bacteria following removal of non-adherent bacteria. After the 45-minute incubation described above, the wells were gently scratched with pipette tips to de-attach the cells from the bottom of each well. Contents in each well was then mixed by gently pipetting up and down. Afterward, aliquots from each well were cultured onto CLED agar. Absence of

bacterial growth on agar following overnight incubation at 37°C confirmed killing of the extracellular bacteria.

The proportion (%) of MRSA that invaded the cells were calculated as follows:

$$\frac{\text{number of bacteria recovered (CFU)}}{\text{number of bacteria inoculated at the start of the experiment}} \times 100$$

(Alsam *et al*., 2006; Yousuf *et al*., 2013).

3.2.4.4. Survival assays

KB skin epithelial and A549 lung cell lines were cultured, grown and MRSA added as described in section 3.2.4. Following the killing of extracellular bacteria (section 3.2.4.3), supernatant from each well was discarded and the wells washed again with PBS. Thereafter, 500 µl of medium appropriate for each cell line were added to each well, and plates were then incubated for 24 hrs at 37°C (in humidified 95% air, 5% CO_2).

3.2.4.4.1. Survival of bacteria in cell culture supernatant

S. aureus can infect, survive in and also lyse host cells; therefore, the percentage of bacteria released by lysed host cells following infection was determined. Following incubation described in section 3.2.4.4, the culture supernatant was gently mixed by pipetting up and down a few times. The number of viable bacteria in the mixed culture supernatant was determined by plating 20 µl (in duplicate) of each well onto plates. The number of colonies on agar was counted on a colony counter (Gallenkamp) after overnight incubation at 37°C.

The proportion (%) of MRSA that was present in the culture supernatant after 24hrs was calculated as follows:

$$\frac{\text{number of bacteria recovered (CFU)}}{\text{number of bacteria inoculated at the start of the experiment}} \times 100$$

3.2.4.4.2. Intracellular survival

To determine the long-term fate of bacteria inside the study cell lines, intracellular survival assays were performed. Following incubation described in section 3.2.4.4, supernatant from each well was discarded and each well was washed once with PBS to remove non-intracellular bacteria. As with association and invasion assays, 150 µl of CelLytic M were added to each well to lyse the cells. 20 µl of lysed solution were cultured onto CLED plates and incubated overnight at 37°C under aerobic conditions. The number of viable bacteria in each well was determined by plating 20µl (in duplicate) of the content of each well onto CLED agar. The number of colonies was counted on a colony counter (Gallenkamp) after overnight incubation at 37ºC.

The proportion (%) of MRSA that survived inside the cells after 24 hrs was calculated as follows:

$$\frac{\text{number of bacteria recovered (CFU)}}{\text{number of bacteria inoculated at the start of the experiment}} \times 100$$

(Alsam *et al*., 2006; Yousuf *et al*., 2013).

3.2.5. Biomass assays

Approximately 10^6 CFU/ml of each MRSA isolate was inoculated in wells of 24-well plates (PBS medium). Plates were then incubated aerobically at 37°C for either 6 or 24 hrs.

3.2.5.1. Biofilm biomass assays

The biofilm biomass was assayed by crystal violet (CV) staining. Each well was washed three times with 500 µl PBS and air-dried. The bacteria were then fixed by adding 250 µl of 99% methanol for 15 mins, following which the methanol was aspirated and the plates allowed to air dry. The wells were then stained with 1% CV solution for 5 min after which excess stain was removed by washing three times with 500 µl PBS. When the wells had dried, the stain was

resolubilised with 160 µl of 33% glacial acetic acid and absorbance was read at an OD of 570 nm using a plate reader (Verhoeven *et al*., 2010).

3.2.6. Statistical analysis

Unless otherwise stated every experiment was repeated 3 times. Experimental data were analysed using a one-tailed unpaired t-test (two-sample unequal variance) for comparison between means. *P* values were considered statistically significant at a 95% confidence interval (i.e. $P < 0.05$). The Mann-Whitney U test was further used for comparison of medians; computed U values were compared to the critical value of U at a confidence interval of 95% / significance value of $P = 0.05$ (U critical = 10 for 6 vs 8 number of samples [One tailed Mann-Whitney U table for critical values: Appendix F]). Difference between the medians of two data sets were considered significant if the computed U value was less than or equal to the critical value (Ucrit).

3.3. Results

In a bid to investigate differences in the interactions of UK and Nigerian MRSA with mammalian cells, interaction assays were performed. Interaction of six UK MRSA (16/11, Nasal, Abdo, Shin, 2 and 3) and eight Nigerian MRSA isolates (GM12, S54N, 114, 193, UM9, 176, 177 and 162) with KB epithelial and A549 lung cell lines were investigated at multiplicity of infection (MOI) 10:1 and MOI 100:1. Unless otherwise stated, means were compared using one-tailed unpaired t-test while medians were compared using the Mann-Whitney U test.

3.3.1. Interaction of MRSA with KB epithelial cell line

3.3.1.1. Association

Results obtained from the association assays showed that on average, the proportion of UK MRSA that associated with KB cells was higher (not significantly) than the proportion of the Nigerian MRSA that associated with KB cells following infection at MOI 10:1 and MOI 100:1 (Figure 3.1, Table 3.1). As can be seen in Table 3.1A, the proportions of individual UK isolates that associated with KB cells at MOI 10:1 ranged from 0.0215% - 0.0575% compared to the 0.0132% - 0.0492% range observed among the Nigerian isolates. Similarly, at MOI 100:1, UK isolates were seen to associate more with the KB cells. This is because the proportions of individual UK isolates that associated with KB cells ranged from 0.0170% - 0.0355% while those of their Nigerian counterparts ranged from 0.0130% - 0.0353% (Table 3.1B). Median values of the proportions of UK MRSA that associated with KB cells were seen to be higher (0.0396 at MOI 10:1 and 0.0253 at MOI 100:1) than those of the Nigerian MRSA (0.0259 at MOI 10:1 and 0.0243 at MOI 100:1) (Table 3.1 A and B). These differences in median values were however seen to be insignificant as the calculated U values at MOIs 10:1 and 100:1, i.e. 13 and 20 respectively, were higher than the critical U value for the data sets (Table 3.1 A and B).

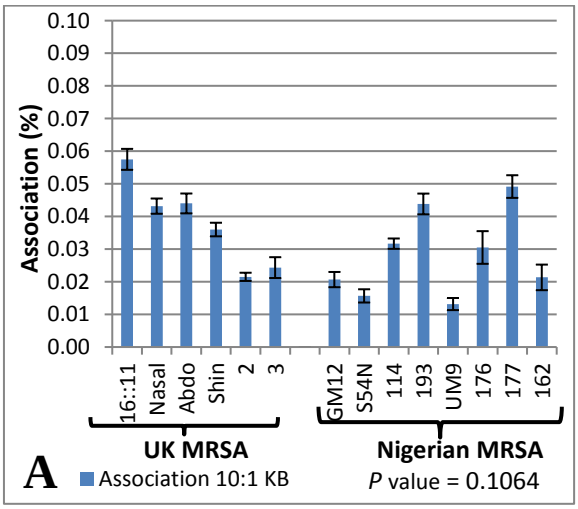
Table 3.1: Proportions of UK and Nigerian MRSA isolates that associated with KB epithelial cells following addition of bacteria at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit i.e. 10 (Mann-Whitney U test).

A

Association, MOI 10:1, KB cells				
UK		Nigerian		
Isolate	**Association (%)**	**Isolate**	**Association (%)**	
16/11	0.0575	**GM12**	0.0207	
Nasal	0.0432	**S54N**	0.0157	***P* value** 0.1064
Abdo	0.0440	**114**	0.0317	
Shin	0.0360	**193**	0.0438	
2	0.0215	**UM9**	0.0132	
3	0.0243	**176**	0.0305	**U value** 13
		177	0.0492	
		162	0.0213	
Mean	**0.0378**	**Mean**	**0.0283**	
Median	**0.0396**	**Median**	**0.0259**	

B

Association, MOI 100:1, KB cells				
UK		Nigerian		
Isolate	**Association (%)**	**Isolate**	**Association (%)**	
16/11	0.0272	**GM12**	0.0162	
Nasal	0.0170	**S54N**	0.0163	***P* value** 0.3667
Abdo	0.0355	**114**	0.0353	
Shin	0.0233	**193**	0.0230	
2	0.0208	**UM9**	0.0130	
3	0.0283	**176**	0.0287	**U value** 20
		177	0.0337	
		162	0.0257	
Mean	**0.0254**	**Mean**	**0.0240**	
Median	**0.0253**	**Median**	**0.0243**	



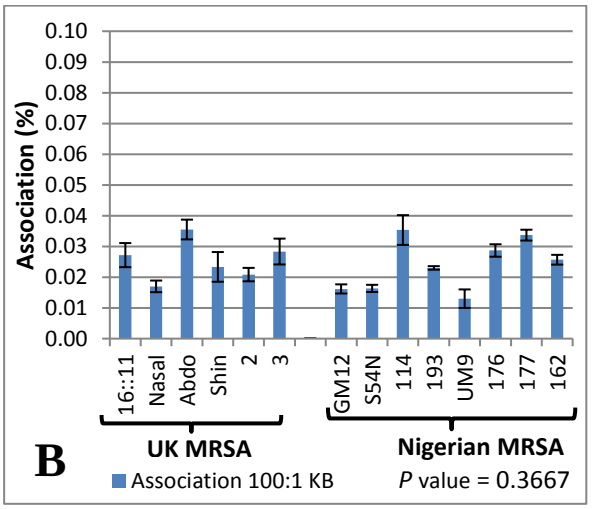


Figure 3.1: Proportions of MRSA isolates that associated with KB epithelial cells following addition of bacteria at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

3.3.1.2. Invasion

Results from this assay revealed that on average, the proportion of Nigerian MRSA that invaded KB epithelial cells was higher (not significantly) than the proportion of invasive UK MRSA at both MOI 10:1 and MOI 100:1 (Figure 3.2, Table 3.2). UK isolate Abdo invaded the KB cells at the highest level (i.e. 0.0385%) amongst both the UK and Nigerian isolates at MOI 10:1 (Table 3.2A). Further to this, another UK isolate (isolate 2) was seen to invade KB cell at the lowest level (i.e. 0.0075%) (Table 3.2A). On the other hand, among the Nigerian isolates at MOI 10:1, isolate 193 invaded KB cells at the highest level (0.0382%) compared with isolate UM9 that invaded the KB cells at the lowest level (Table 3.2A). The results showed that the range of proportions of UK isolates that invaded the KB cells (at MOI 100:1) was higher (i.e. 0.0062% - 0.0187%) than that of their Nigerian counterparts (i.e. 0.0036% - 0.0148%) (Table 3.2B). However, the mean of the proportions of the Nigerian isolates that invaded the KB cells was higher (0.0111%) than that of their UK counterparts (0.0109%) (Table 3.2B). Similar to the results obtained from the t test, results from the Mann-Whitney U test comparison of medians of also showed that no significant differences existed between the proportions of UK and Nigerian MRSA that invaded KB cells at MOIs 10:1 and 100:1 (Table 3.2). This is because the calculated U value of the differences between the medians at MOI 10:1 was 22, while that at 100:1 was 24 (Table 3.2 A and B); as both were higher than the critical U value for the data sets, the observed differences between the medians at both MOIs were insignificant.

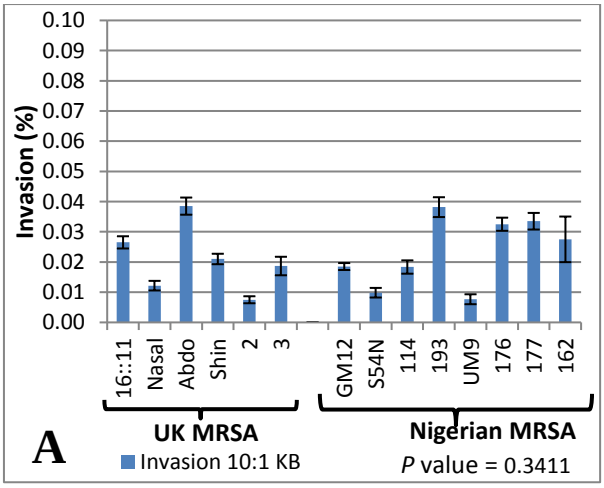
Table 3.2: Proportions of UK and Nigerian MRSA isolates that invaded KB epithelial cells following infection at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit i.e. 10 (Mann-Whitney U test).

A

Invasion, MOI 10:1, KB cells				
UK		Nigerian		
Isolate	**Invasion (%)**	**Isolate**	**Invasion (%)**	
16/11	0.0265	**GM12**	0.0185	***P* value** 0.3411
Nasal	0.0122	**S54N**	0.0098	
Abdo	0.0385	**114**	0.0183	
Shin	0.0210	**193**	0.0382	
2	0.0075	**UM9**	0.0077	**U value** 22
3	0.0187	**176**	0.0325	
		177	0.0335	
		162	0.0275	
Mean	**0.0207**	**Mean**	**0.0233**	
Median	**0.0198**	**Median**	**0.0230**	

B

Invasion, MOI 100:1, KB cells				
UK		Nigerian		
Isolate	**Invasion (%)**	**Isolate**	**Invasion (%)**	
16/11	0.0187	**GM12**	0.0090	***P* value** 0.4812
Nasal	0.0078	**S54N**	0.0095	
Abdo	0.0138	**114**	0.0137	
Shin	0.0097	**193**	0.0120	
2	0.0062	**UM9**	0.0036	**U value** 24
3	0.0095	**176**	0.0148	
		177	0.0137	
		162	0.0122	
Mean	**0.0109**	**Mean**	**0.0111**	
Median	**0.0096**	**Median**	**0.0121**	



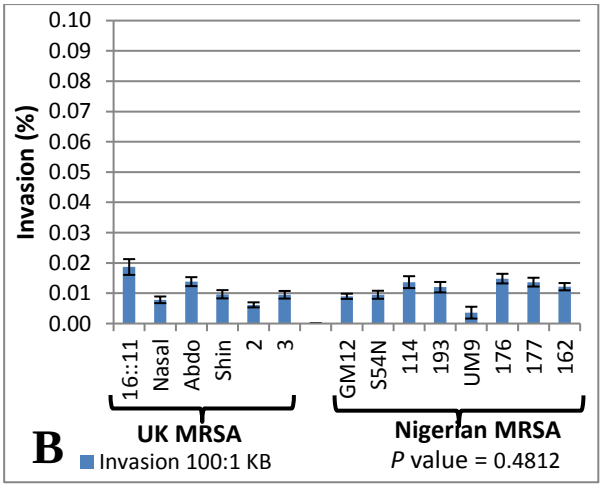


Figure 3.2: Proportions of MRSA isolates that invaded KB epithelial cells at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

3.3.1.3. Survival

Analysis of the results from this study shows that there is no significant difference between the mean percentages of UK and Nigerian MRSA that survived in KB epithelial cells (survival in culture supernatant, intracellular survival and total survival) following infection at MOIs 10:1 and 100:1 (Tables 3.3 – 3.5; Figures 3.3 – 3.5).

3.3.1.3.1. Survival of bacteria in cell culture supernantant: Results showed that at MOI 10:1, the Nigerian isolate UM9 had the highest level of survival in the culture supernatant (19.83%) compared to all other isolates (UK and Nigerian) (Table 3.3A). However, it was UK isolate Abdo that had the lowest level of survival in the culture supernatant (1.22%) among all the MRSA isolates (at MOI 10:1) (Table 3.3A). Though it was seen that the mean percentage of Nigerian MRSA that survived in the culture supernatant was higher than the mean percentage of their UK counterparts 24 hrs post infection (MOI 10:1), the difference was not significant (Figure 3.3A, Table 3.3A). At MOI 100:1, the range of the proportions of the UK isolates that survived in the culture supernatant post infection, i.e. 0.32% - 1.45%, was higher than that of the Nigerian isolates, i.e. 0.22% - 1.43% (Table 3.3B). However, even though the UK isolates survived at a higher level than the Nigerian isolates in the supernatant, the difference was insignificant (Figure 3.3B, Table 3.3B). Medians of the proportions of UK MRSA that survived in the culture supernatant were seen to be higher (5.967 at MOI 10:1 and 0.80 at MOI 100:1) than those of their Nigerian counterparts (5.333 at MOI 10:1 and 0.47 at MOI 100:1). The U values calculated using the Mann-Whitney U test for medians at MOIs 10:1 and 100:1 were 17 and 18 respectively (Table 3.3 A and B). As these values are higher than the critical U value for the data sets, it is an indication that there are no significant differences between the medians of the proportions of UK and Nigerian MRSA that survived in the culture supernatant 24 hrs post infection.

Table 3.3: Proportions of UK and Nigerian MRSA isolates that survived in the culture supernatant of KB epithelial cells after 24 hrs following infection at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was ≤ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Survival of Bacteria in Cell Culture Supernatant 10:1 KB				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	2.43	**GM12**	5.83	
Nasal	13.67	**S54N**	2.75	***P* value** 0.2910
Abdo	1.22	**114**	4.78	
Shin	9.67	**193**	4.83	
2	9.50	**UM9**	19.83	**U value** 17
3	1.48	**176**	4.13	
		177	9.83	
		162	12.00	
Mean	**6.328**	**Mean**	**8.000**	
Median	**5.967**	**Median**	**5.333**	

B

Survival of Bacteria in Cell Culture Supernatant 100:1 KB				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	0.69	**GM12**	0.48	
Nasal	1.45	**S54N**	0.43	***P* value** 0.1961
Abdo	0.26	**114**	0.34	
Shin	1.18	**193**	0.86	
2	0.92	**UM9**	0.50	**U value** 18
3	0.32	**176**	0.22	
		177	0.47	
		162	1.43	
Mean	**0.80**	**Mean**	**0.59**	
Median	**0.80**	**Median**	**0.47**	

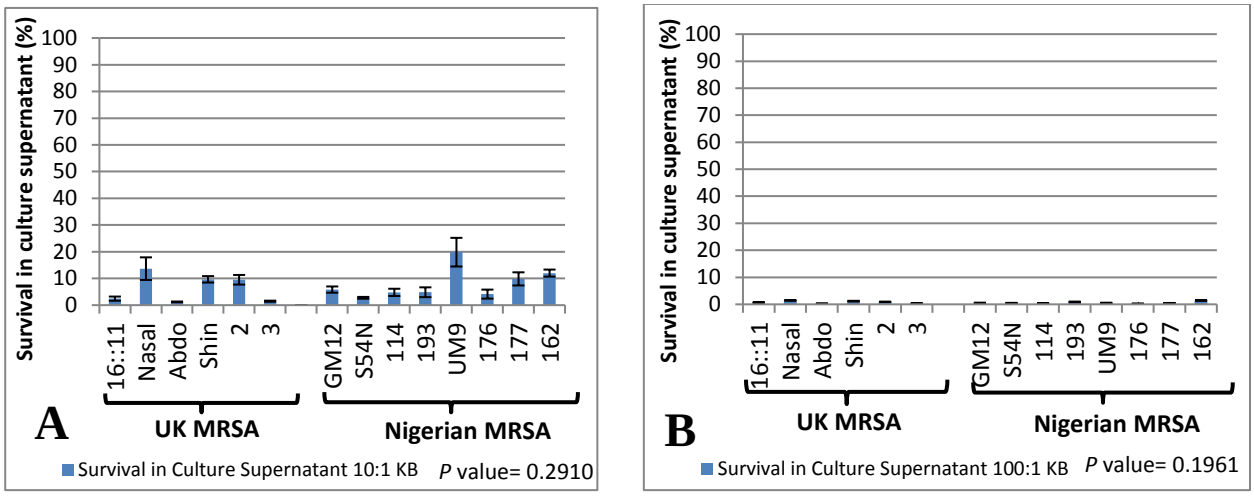


Figure 3.3: Proportions of MRSA isolates that survived in the culture supernatant of KB epithelial cells 24 hrs post infection at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

Survival of bacteria in cell culture supernatant = Survival in culture supernatant

3.3.1.3.2. Intracellular survival: Results from the intracellular survival assays shows the range percentage survival of Nigerian isolates inside the KB cells to be wider at both MOIs 10:1 (18.67% - 96.67%) and 100:1 (1.38% - 11.67%) compared with their UK counterparts (i.e. 5.33% - 66.67% at MOI 10:1 and 2.07% - 8.17% at MOI 100:1) (Table 3.4). Nevertheless, there was no significant difference in the mean percentage intracellular survival between the UK and Nigerian isolates (Figure 3.4, Table 3.4). The results showed that although the median of the proportions of UK MRSA that survived intracellularly was higher (46.33) than that of Nigerian MRSA (44.42) at MOI 10:1, the opposite was the case at MOI 100:1 i.e. median for UK MRSA was 3.77% while that for Nigerian MRSA was 6.99%. A comparison of these medians showed that the calculated U value at MOI 10:1 was 21 (> 10 i.e. U critical) (Table 3.4A), an indication that there was no significant difference between the proportions of UK and Nigerian MRSA that survived intracellularly in the KB cells 24 hrs post infection. This was not the case at MOI 100:1, as the calculated U value of 3 (Table 3.4B) was less than the critical U value for the data sets i.e. 10, i.e. the median value of the proportions of Nigerian MRSA that survived intracellularly is significantly higher than that of their UK counterparts.

Table 3.4: Proportions of UK and Nigerian MRSA isolates that survived inside the KB epithelial cells after 24 hrs following infection at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Intracellular Survival 10:1 KB				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	28.67	**GM12**	96.67	***P* value** 0.1946
Nasal	48.00	**S54N**	45.83	
Abdo	66.67	**114**	43.00	
Shin	52.00	**193**	33.67	
2	44.67	**UM9**	90.00	
3	5.33	**176**	18.67	**U value** 21
		177	63.67	
		162	30.33	
Mean	**40.89**	**Mean**	**52.73**	
Median	**46.33**	**Median**	**44.42**	

B

Intracellular Survival 100:1 KB				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	2.07	**GM12**	4.68	***P* value** 0.0592
Nasal	4.03	**S54N**	3.68	
Abdo	3.33	**114**	5.32	
Shin	8.17	**193**	10.17	
2	4.65	**UM9**	11.67	
3	3.50	**176**	1.38	**U value** 3
		177	9.50	
		162	8.67	
Mean	**4.29**	**Mean**	**6.88**	
Median	**3.77**	**Median**	**6.99**	

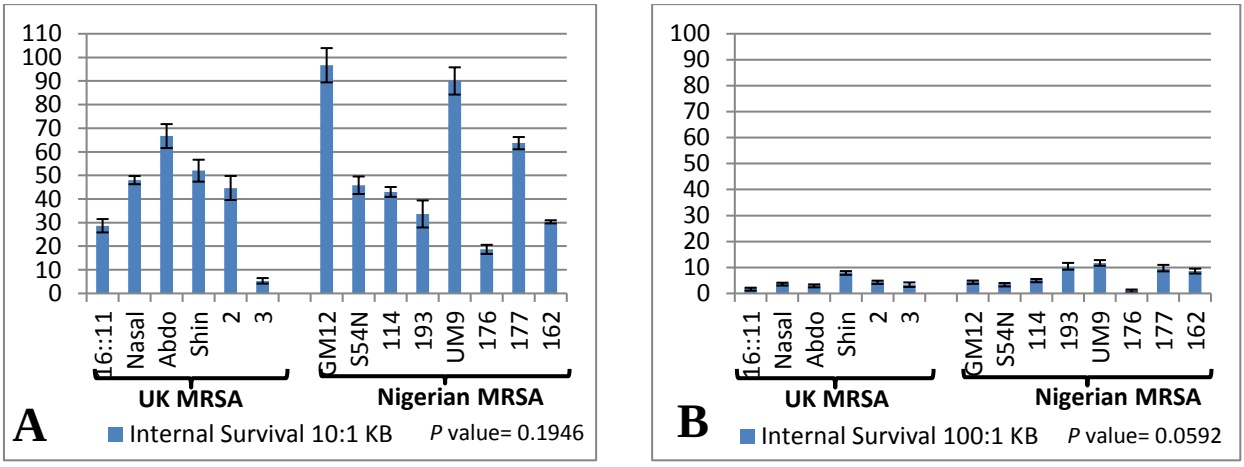


Figure 3.4: Proportions of MRSA isolates that survived inside the KB epithelial cells 24 hrs post infection at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

Intracellular survival = Internal survival

3.3.1.3.3. Total survival: Total survival was determined by the addition of the survival of bacteria in cell culture supernatant and intracellular survival proportions. Based on this, Nigerian isolate UM9 was seen to have the highest level of survival at both MOI 10:1 (109.83%) and 100:1 (12.16%) among all the isolates (both UK and Nigerian) (Table 3.5). Nigerian isolate 176 was seen to have the least level of survival among the Nigerian isolates at both MOI 10:1 (22.80%) and 100:1 (1.61%) (Table 3.5). While UK isolates Abdo and 3 had the highest and lowest levels of survival (67.89% and 6.82% respectively) among the UK isolates at MOI 10:1 (Table 3.5A), it was isolates Shin and 16/11 that had the highest and lowest levels of survival at MOI 100:1 (9.35% and 2.76% respectively) (Table 3.5B). On average, the percentage total survival of Nigerian MRSA in KB cells (at both MOI 10:1 and 100:1) was not significantly higher than the percentage total survival of UK MRSA in KB cells (Table 3.5, Figure 3.5). No significant differences were also observed in the differences between the medians. As seen in Table 3.5, the calculated U values for the total survival of UK and Nigerian MRSA 24 hrs following infection at MOIs 10:1 and 100:1 were 21 and 13 (respectively), both of which are higher than the critical U value of the data sets (10).

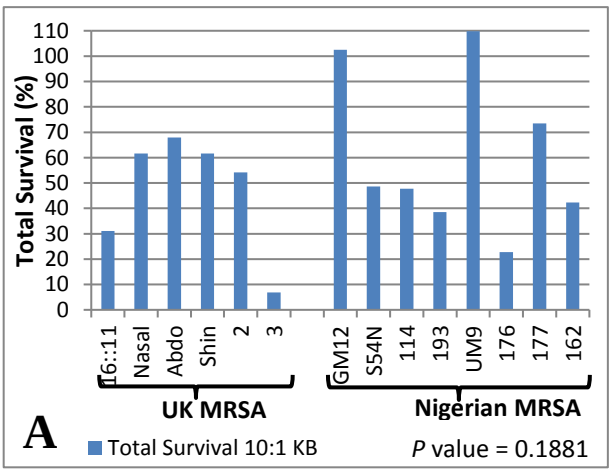
Table 3.5: Sum total of the proportions of UK and Nigerian MRSA isolates that survived both inside and in the culture supernatant of KB epithelial cells after 24 hrs following infection at MOI 10:1 **(A)**, and MOI 100:1 **(B)**. Results are the sum of percentage survival of bacteria in cell culture supernatant and percentage intracellular survival. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Total Survival 10:1 KB				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	31.10	**GM12**	102.50	
Nasal	61.67	**S54N**	48.58	***P* value** 0.1881
Abdo	67.88	**114**	47.78	
Shin	61.67	**193**	38.50	
2	54.17	**UM9**	109.83	
3	6.82	**176**	22.80	**U value** 21
		177	73.50	
		162	42.33	
Mean	**47.22**	**Mean**	**60.73**	
Median	**57.93**	**Median**	**48.18**	

B

Total Survival 100:1 KB				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	2.76	**GM12**	5.16	
Nasal	5.48	**S54N**	4.11	***P* value** 0.0887
Abdo	3.59	**114**	5.66	
Shin	9.35	**193**	11.03	
2	5.57	**UM9**	12.16	
3	3.82	**176**	1.61	**U value** 13
		177	9.97	
		162	10.10	
Mean	**5.10**	**Mean**	**7.48**	
Median	**4.6525**	**Median**	**7.8125**	



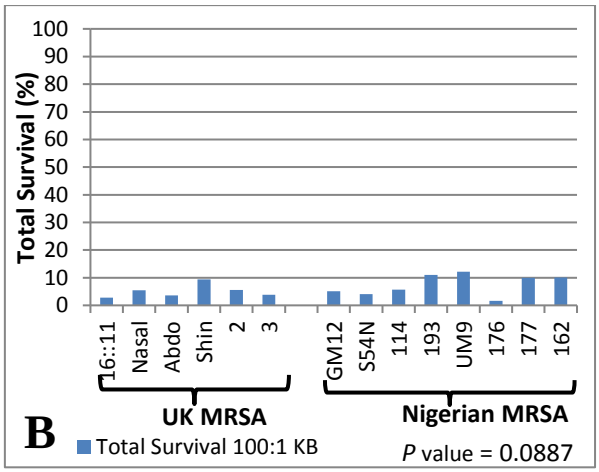


Figure 3.5: Sum total of the proportions of UK and Nigerian MRSA isolates that survived both inside and in the cell culture supernatant of KB epithelial cells after 24 hrs following infection at MOI 10:1 **(A)**, and MOI 100:1 **(B)**. Results are the sum of percentage survival of bacteria in cell culture supernatant and percentage intracellular survival. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

3.3.2. Interaction of MRSA with A549 lung cell line

3.3.2.1. Association

The results show that among the UK isolates, isolate 2 associated with A549 cells the least at MOIs 10:1 (0.025%) and 100:1 (0.0113%) (Table 3.6). Isolate 16/11 was seen to associate with A549 cells at the highest level at MOI 10:1 (0.0450%) while at MOI 100:1, isolate Shin associated with A549 cells at the highest level among the UK isolates (Table 3.6). Nigerian isolate 177 associated with A549 cells at the highest level at MOIs 10:1 (0.0452%) and 100:1 (0.0268%) among the Nigerian isolates (Table 3.6). However, isolate UM9 had the lowest level of association with the A549 cells at MOI 10:1 (Table 3.6A) while isolate S54N had the lowest level of association with A549 cells at MOI 100:1 among the Nigerian isolates (Table 3.6B). A comparison of means showed that a higher percentage of UK MRSA associated with A549 lung cells at MOI 10:1 and MOI 100:1 compared to their Nigerian counterparts (Table 3.6). These differences in association of UK and Nigerian MRSA with A549 cells were insignificant at both MOIs (Figure 3.6, Table 3.6). A further comparison of medians showed that the median values of the proportions of UK isolates that associated with A549 cells were higher (0.0293 at MOI 10:1; and 0.0184 at MOI 100:1) than the median values of their Nigerian counterparts (0.0268 at MOI 10:1 and 0.0149 at MOI 100:1) (Table 3.6). However, as the calculated U values at both MOIs (20 at MOI 10:1 and 21 at MOI 100:1) were higher than the U critical value (10), the observed differences in median is insignificant.

Table 3.6: Proportions of UK and Nigerian MRSA isolates that associated with A549 cells following addition of bacteria at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Association 10:1 A549				
UK		**Nigerian**		
Isolate	**Association (%)**	**Isolate**	**Association (%)**	
16/11	0.0450	**GM12**	0.0258	***P* value** 0.2620
Nasal	0.0288	**S54N**	0.0313	
Abdo	0.0293	**114**	0.0273	
Shin	0.0393	**193**	0.0262	
2	0.0250	**UM9**	0.0150	**U value** 20
3	0.0255	**176**	0.0388	
		177	0.0452	
		162	0.0230	
Mean	**0.0322**	**Mean**	**0.0291**	
Median	**0.0293**	**Median**	**0.0268**	

B

Association 100:1 A549				
UK		**Nigerian**		
Isolate	**Association (%)**	**Isolate**	**Association (%)**	
16/11	0.0215	**GM12**	0.0142	***P* value** 0.3221
Nasal	0.0140	**S54N**	0.0103	
Abdo	0.0222	**114**	0.0188	
Shin	0.0227	**193**	0.0133	
2	0.0113	**UM9**	0.0092	**U value** 21
3	0.0153	**176**	0.0232	
		177	0.0268	
		162	0.0157	
Mean	**0.0178**	**Mean**	**0.0164**	
Median	**0.0184**	**Median**	**0.0149**	

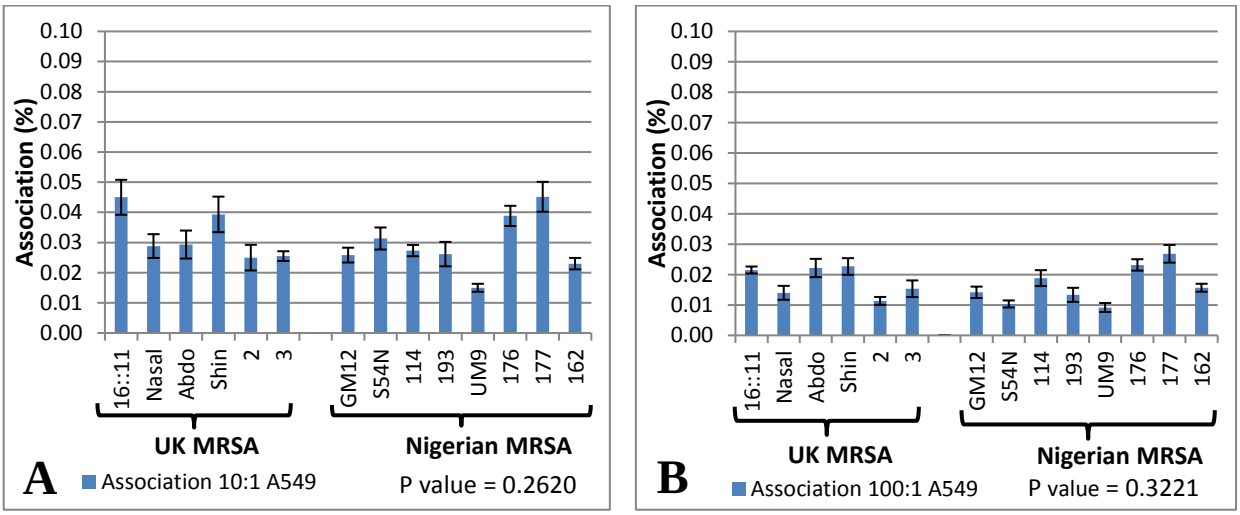


Figure 3.6: Proportions of MRSA isolates that associated with A549 lung cells following addition of bacteria at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

3.3.2.2. Invasion

The results show that of all the UK isolates, UK isolate Shin and 2 invaded the A549 cells at the highest (0.0225%) and lowest (0.0097%) levels respectively at MOI 10:1 (Table 3.7A). On the other hand, it was UK isolates 16/11 and Nasal invaded the A549 cells at the highest and lowest levels (0.0140% and 0.0030% respectively) at MOI 100:1 among the UK isolates (Table 3.7B). However, among the Nigerian isolates, isolates 176 and UM9 were seen to invade the A549 cells at the highest and lowest rates at both MOI 10:1 (0.0225% and 0.0053% respectively) and 100:1 (0.0168% and 0.0033% respectively) (Table 3.7).

Based on the results obtained, it was seen that on average a higher proportion of UK MRSA invaded A549 lung cells than Nigerian MRSA at MOI 10:1 (Table 3.7A); the reverse was however the case at MOI 100:1 (Table 3.7B). In both instances, it was seen that these observed differences in invasion were not significant (Figure 3.7, Table 3.7). The observed differences in medians of the proportions at which UK and Nigerian MRSA invaded A549 cells were insignificant. Median values of the invading proportions of Nigerian MRSA were higher (0.0149 at MOI 10:1 and 0.0089 at MOI 100:1) than those of their UK counterparts (0.0122 at MOI 10:1 and 0.0086 at MOI 100:1) (Table 3.7). Nevertheless, the calculated U values (24 at MOI 10:1 and 21 at MOI 100:1) were higher than the critical U value, thereby making the differences between the medians insignificant.

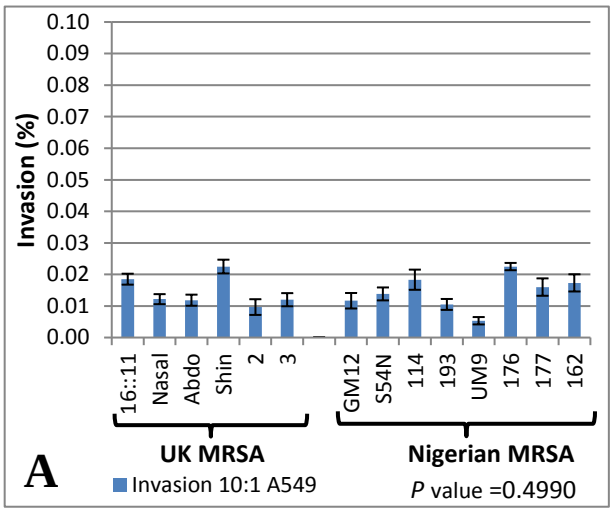
Table 3.7: Proportions of UK and Nigerian MRSA isolates that invaded A549 cells following infection at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was < to Ucrit i.e. 10 (Mann-Whitney U test).

A

Invasion 10:1 A549				
UK		Nigerian		
Isolate	**Invasion (%)**	**Isolate**	**Invasion (%)**	
16/11	0.0185	**GM12**	0.0117	
Nasal	0.0122	**S54N**	0.0138	***P* value** 0.4990
Abdo	0.0118	**114**	0.0183	
Shin	0.0225	**193**	0.0105	
2	0.0097	**UM9**	0.0053	
3	0.0120	**176**	0.0225	**U value** 24
		177	0.0160	
		162	0.0173	
Mean	**0.014444**	**Mean**	**0.014438**	
Median	**0.0122**	**Median**	**0.0149**	

B

Invasion 100:1 A549				
UK		Nigerian		
Isolate	**Invasion (%)**	**Isolate**	**Invasion (%)**	
16/11	0.0140	**GM12**	0.0067	
Nasal	0.0030	**S54N**	0.0048	***P* value** 0.3226
Abdo	0.0086	**114**	0.0112	
Shin	0.0112	**193**	0.0073	
2	0.0033	**UM9**	0.0033	
3	0.0086	**176**	0.0168	**U value** 21
		177	0.0105	
		162	0.0132	
Mean	**0.0081**	**Mean**	**0.0092**	
Median	**0.0086**	**Median**	**0.0089**	



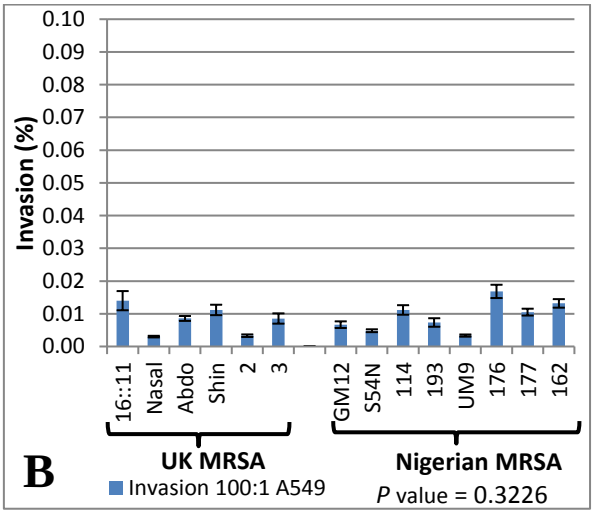


Figure 3.7: Proportions of MRSA isolates that invaded A549 lung cells at MOI 10:1 (**A**) and MOI 100:1 (**B).** Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

3.3.2.3. Survival

As seen in the association and survival assays, the differences observed between survival of UK and Nigerian MRSA isolates were insignificant (Tables 3.8 – 3.10, Figures 3.8 – 3.10).

3.3.2.3.1. Survival of bacteria in cell culture supernatant: Results from the survival assays show that among all the isolates, it was Nigerian isolates that survived at the highest levels in the culture supernatants at MOI 10:1 (isolate UM9 = 2.78%; Table 3.8A) and MOI 100:1 (isolate 162 = 25.17%; Table 3.8B). Regardless, it was seen that on average, the level of survival of UK MRSA in the culture supernatant was higher at both MOI 10:1 (0.77%; Table 3.8A) and MOI 100:1 (11.74%; Table 3.8B). The median values of the proportions of UK MRSA that survived in cell culture supernatant 24 hrs post infection were higher (13.00 at MOI 10:1 and 0.70 at MOI 100:1) that those of their Nigerian counterparts (10.41 at MOI 10:1 and 0.52 at MOI 100:1). These differences were however not significant as the calculated U values at MOIs 10:1 (21) and 100:1 (23), were higher than the critical U value (10).

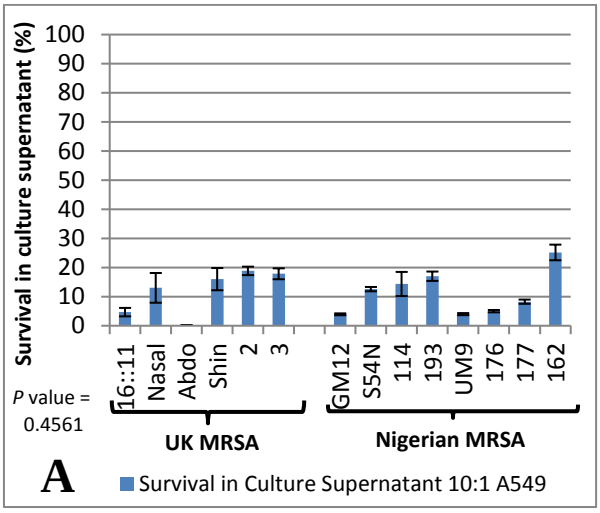
Table 3.8: Proportions of UK and Nigerian MRSA isolates that survived in the culture supernatant of A549 cells after 24 hrs following infection at MOI 10:1 **(A)** and MOI 100:1 **(B)**. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Survival of Bacteria in Cell Culture Supernatant 10:1 A549				
UK		**Nigerian**		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	4.67	**GM12**	3.92	
Nasal	13.00	**S54N**	12.57	***P* value** 0.4561
Abdo	0.10	**114**	14.33	
Shin	16.00	**193**	17.00	
2	18.83	**UM9**	3.98	
3	17.83	**176**	5.00	**U value** 21
		177	8.25	
		162	25.17	
Mean	**11.74**	**Mean**	**11.28**	
Median	**13.00**	**Median**	**10.41**	

B

Survival of Bacteria in Cell Culture Supernatant 100:1 A549				
UK		**Nigerian**		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	0.16	**GM12**	0.25	
Nasal	1.38	**S54N**	0.57	***P* value** 0.4943
Abdo	0.31	**114**	0.68	
Shin	1.08	**193**	0.37	
2	1.50	**UM9**	2.78	
3	0.16	**176**	0.14	**U value** 23
		177	0.47	
		162	0.81	
Mean	**0.77**	**Mean**	**0.76**	
Median	**0.70**	**Median**	**0.52**	



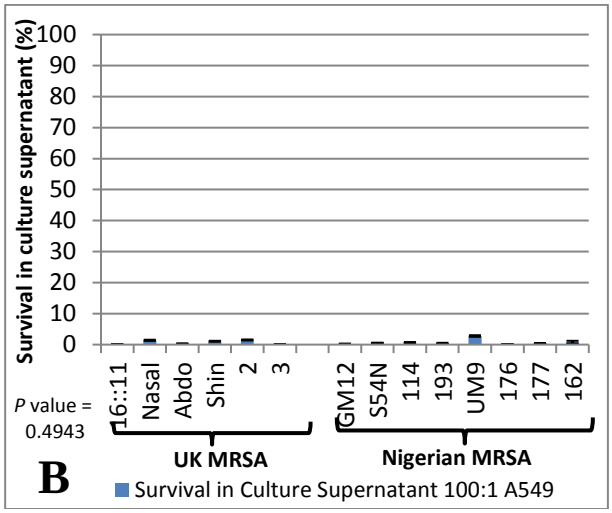


Figure 3.8: Proportions of MRSA isolates that survived in the culture supernatant of A549 cells 24 hrs post infection at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the unpaired one-tailed t test assuming unequal variance.

Survival of bacteria in cell culture supernatant = Survival in culture supernatant

3.3.2.3.2. Intracellular survival: Overall, UK MRSA isolates were seen to survive inside the A549 cells at a lower level compared with the Nigerian MRSA (significantly at MOI 10:1 and insignificantly at MOI 100:1) (Figure 3.9, Table 3.9). As can be seen in Table 3.9A, percentage intracellular survival of individual UK MRSA isolates ranged from 7.33% - 87.0% at MOI 10:1, which is lower than what was observed among the Nigerian MRSA (48.50% - 91.83%). Similarly, at MOI 100:1, the percentage survival of each UK MRSA inside A549 cells ranged from 2.00% - 11.33%, which was a lower range compared to that of their Nigerian counterparts (5.33% - 14.83%) (Table 3.9B). Although the median values of the proportions of Nigerian MRSA that survived intracellularly were higher (60.50 at MOI 10:1 and 10.92 at MOI 100:1) than those of the UK MRSA (58.00 at MOI 10:1 and 7.88 at MOI 100:1), the differences were insignificant as the calculated U values at MOIs 10:1 (19) and 100:1 (14) were higher than the critical U values for the data sets.

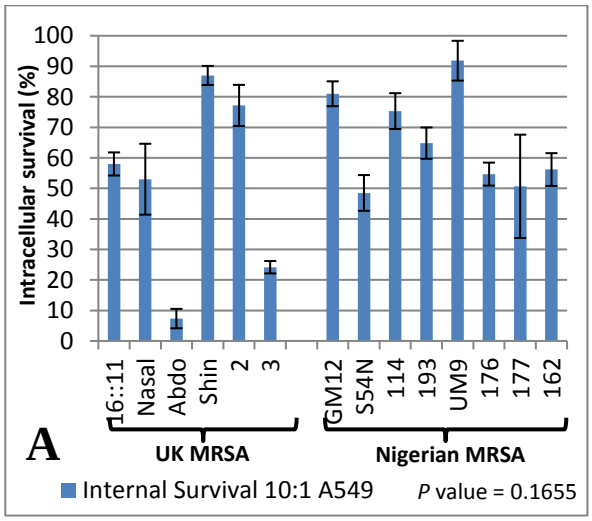
Table 3.9: Proportions of UK and Nigerian MRSA isolates that survived inside the A549 cells after 24 hrs following infection at MOI 10:1 **(A)** and MOI 100:1 **(B).** Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Intracellular Survival 10:1 A549				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	58.00	**GM12**	81.00	***P* value** 0.1655
Nasal	53.00	**S54N**	48.50	
Abdo	7.33	**114**	75.33	
Shin	87.00	**193**	64.83	
2	77.17	**UM9**	91.83	**U value** 19
3	24.17	**176**	54.67	
		177	50.67	
		162	56.17	
Mean	**51.11**	**Mean**	**65.38**	
Median	**58.00**	**Median**	**60.50**	

B

Intracellular Survival 100:1 A549				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	2.00	**GM12**	13.50	***P* value** 0.0460
Nasal	8.83	**S54N**	6.55	
Abdo	2.42	**114**	14.83	
Shin	11.33	**193**	7.90	
2	7.23	**UM9**	13.50	**U value** 14
3	8.52	**176**	5.33	
		177	13.00	
		162	8.83	
Mean	**6.72**	**Mean**	**10.43**	
Median	**7.88**	**Median**	**10.92**	



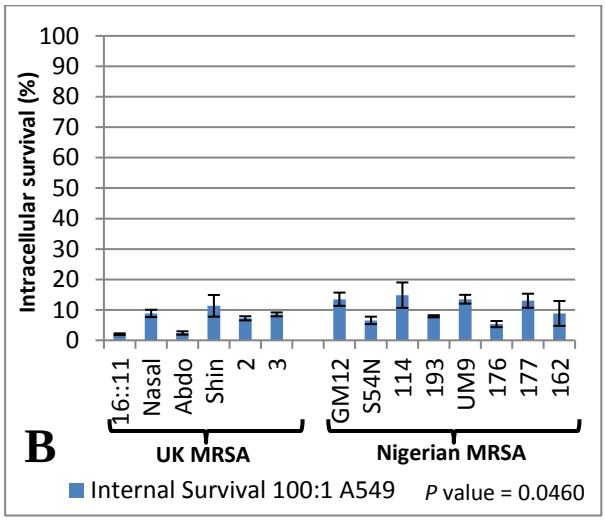


Figure 3.9: Proportions of MRSA isolates that survived inside the A549 cells 24 hrs post infection at MOI 10:1 (**A**) and MOI 100:1 (**B**). Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance.

Intracellular survival = Internal survival

3.3.2.3.3. Total survival: Total survival was determined by the addition of the survival of bacteria in cell culture supernatant and intracellular survival proportions. Thus, it was seen that UK isolate Shin and Nigerian isolate UM9 survived at the highest levels at MOIs 10:1 and 100:1 among the UK and Nigerian isolates respectively (Table 3.10). While isolate Abdo had the lowest level of total survival at MOI 10:1 (7.43%) among the UK isolates (Table 3.10A), UK isolate had the least level of total survival at MOI 100:1 (2.16%) (Table 3.10B). Among the Nigerian isolates, isolate 177 had the least level of total survival at MOI 10:1 (58.92%) (Table 3.10A), while isolate 176 had the least level of total survival at MOI 100:1 (5.47%) (Table 3.10B). In addition, the average percentage of Nigerian MRSA that survived (total survival) in A549 cells following invasion was higher than the average percentage survival (total survival) of their UK counterparts following infection at MOIs 10:1 and 100:1 (Figure 3.10, Table 3.10). Insignificant differences were observed in the sum total of proportions of UK and Nigerian MRSA that survived in the A549 cells 24 hrs post infection. This is because the calculated U values were higher (22 at MOI 10:1 and 14 at MOI 100:1) than the critical U values for both data sets.

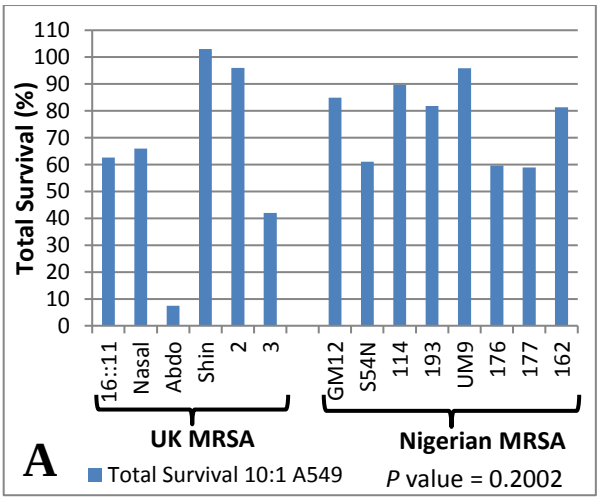
Table 3.10: Sum total of the proportions of UK and Nigerian MRSA isolates that survived both inside and in the culture supernatant of KB epithelial cells after 24 hrs following infection at MOI 10:1 **(A),** and MOI 100:1 **(B)**. Results are the sum of percentage survival of bacteria in cell culture supernatant and percentage intracellular survival. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

A

Total Survival 10:1 A549				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	62.67	**GM12**	84.92	
Nasal	66.00	**S54N**	61.07	***P* value** 0.2002
Abdo	7.43	**114**	89.67	
Shin	103.00	**193**	81.83	
2	96.00	**UM9**	95.82	
3	42.00	**176**	59.67	**U value** 22
		177	58.92	
		162	81.33	
Mean	**62.85**	**Mean**	**76.65**	
Median	**66.00**	**Median**	**51.58**	

B

Total Survival 100:1 A549				
UK		Nigerian		
Isolate	**Survival (%)**	**Isolate**	**Survival (%)**	
16/11	2.16	**GM12**	13.75	
Nasal	10.22	**S54N**	7.13	***P* value** 0.0622
Abdo	2.73	**114**	15.52	
Shin	12.42	**193**	8.27	
2	8.73	**UM9**	16.28	
3	8.68	**176**	5.47	**U value** 14
		177	13.47	
		162	9.64	
Mean	**7.49**	**Mean**	**11.19**	
Median	**8.70**	**Median**	**11.56**	



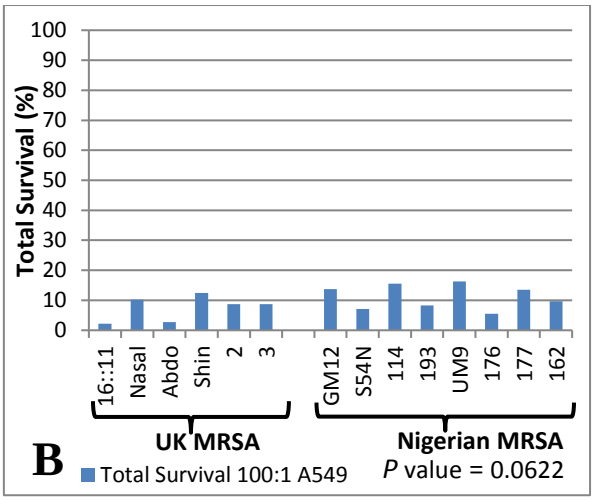


Figure 3.10: Sum total of the proportions of UK and Nigerian MRSA isolates that survived both inside and in the culture supernatant of KB epithelial cells after 24 hrs following infection at MOI 10:1 **(A),** and MOI 100:1 **(B)**. Results are the sum of percentage survival of bacteria in cell culture supernatant and percentage intracellular survival. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance**.**

The results showed that the level of association of the UK isolates was higher with the KB epithelial cell line than with the A549 lung cell line at both MOI 10:1 (KB – 0.0378%; A549 – 0.0322%) and 100:1 (KB – 0.0254%; A549 – 0.0178%) (Table 3.11). This difference in level of association was however only significant at MOI 100:1 i.e. *P* value = 0.023. With regards the level of invasion, although the results showed that the UK MRSA invaded the KB epithelial cells at a higher level than they did the A549 cell line, the differences in level of invasion was not significant at either MOIs (MOI 10:1 [*P* value = 0.115]; MOI 100:1 [*P* value = 0.147]) (Table 3.11). When it came to the level of survival, the results showed that the UK MRSA survived at a higher level inside the A549 lung cells (MOI 10:1 – 62.85%; MOI 100:1 – 7.49%) than they did inside the KB epithelial cell lines (MOI 10:1 – 47.22%; MOI 100:1 – 5.10%) (Table 3.11).

Table 3.11: Proportions of UK MRSA that interacted with KB skin and A549 lung cell lines following infection at MOIs 10:1 and 100:1. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test. Significant *P* values are highlighted in grey.

UK Means Compared (%)							
MOI 10:1				**MOI 100:1**			
	KB	**A549**	***P* values**		**KB**	**A549**	***P* values**
Association							
16/11	0.0575	0.0450	0.202	**16/11**	0.0272	0.0215	0.023
Nasal	0.0432	0.0288		**Nasal**	0.0170	0.0140	
Abdo	0.0440	0.0293		**Abdo**	0.0355	0.0222	
Shin	0.0360	0.0393		**Shin**	0.0233	0.0227	
2	0.0215	0.0250		**2**	0.0208	0.0113	
3	0.0243	0.0255		**3**	0.0283	0.0153	
Mean	**0.0378**	**0.0322**		**Mean**	**0.0254**	**0.0178**	
Invasion							
16/11	0.0265	0.0185	0.115	**16/11**	0.0187	0.0140	0.147
Nasal	0.0122	0.0122		**Nasal**	0.0078	0.0030	
Abdo	0.0385	0.0118		**Abdo**	0.0138	0.0086	
Shin	0.0210	0.0225		**Shin**	0.0097	0.0112	
2	0.0075	0.0097		**2**	0.0062	0.0033	
3	0.0187	0.0120		**3**	0.0095	0.0086	
Mean	**0.0207**	**0.014444**			**0.0109**	**0.0081**	
Total Survival							
16/11	31.10	62.67	0.194	**16/11**	2.76	2.16	0.123
Nasal	61.67	66.00		**Nasal**	5.48	10.22	
Abdo	67.88	7.43		**Abdo**	3.59	2.73	
Shin	61.67	103.00		**Shin**	9.35	12.42	
2	54.17	96.00		**2**	5.57	8.73	
3	6.82	42.00		**3**	3.82	8.68	
Mean	**47.22**	**62.85**		**Mean**	**5.10**	**7.49**	

3.3.4. Interaction efficiencies of Nigerian MRSA with KB and A549 cell lines: comparisons

The results showed that Nigerian MRSA isolates associated with the KB cells at a significantly higher level (0.0240%) than they did with the A549 lung cell line (0.0164%) at MOI 100:1; the opposite was the case at MOI 10:1 (KB – 0.0283%; A549 – 0.0291%) (Table 3.12). Though Nigerian MRSA invaded the KB epithelial cells at a higher level (10:1 – 0.0233%; 100:1 – 0.0111%) than they did the A549 cells (10:1 – 0.0144%; 100:1 – 0.0092%), these observed differences were not significant i.e. *P* values < 0.05 (Table 3.12). Similar to what was obtained in the case of the UK MRSA, the Nigerian MRSA were seen to survive at a higher level within the A549 lung cells (10:1 – 76.65%; 100:1 – 11.190%) than within the KB cells (10:1 – 60.73%; 100:1 – 7.48%) (Table 3.12). However, in this case, the level of survival within the A549 cells was significantly higher than the level of survival within the KB cells at MOI 100:1 (*P* value = 0.040).

Table 3.12: Proportions of Nigerian MRSA that interacted with KB skin and A549 lung cell lines following infection at MOIs 10:1 and 100:1. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test. Significant *P* values are highlighted in grey.

Nigerian Means Compared (%)							
MOI 10:1				**MOI 100:1**			
	KB	**A549**	***P* values**		**KB**	**A549**	***P* values**
Association							
GM12	0.0207	0.0258		**GM12**	0.0162	0.0142	
S54N	0.0157	0.0313		**S54N**	0.0163	0.0103	
114	0.0317	0.0273		**114**	0.0353	0.0188	
193	0.0438	0.0262		**193**	0.0230	0.0133	
UM9	0.0132	0.0150	0.443	**UM9**	0.0130	0.0092	0.029
176	0.0305	0.0388		**176**	0.0287	0.0232	
177	0.0492	0.0452		**177**	0.0337	0.0268	
162	0.0213	0.0230		**162**	0.0257	0.0157	
Mean	**0.0283**	**0.0291**		**Mean**	**0.0240**	**0.0164**	
Invasion							
GM12	0.0185	0.0117		**GM12**	0.0090	0.0067	
S54N	0.0098	0.0138		**S54N**	0.0095	0.0048	
114	0.0183	0.0183		**114**	0.0137	0.0112	
193	0.0382	0.0105		**193**	0.0120	0.0073	
UM9	0.0077	0.0053	0.033	**UM9**	0.0036	0.0033	0.194
176	0.0325	0.0225		**176**	0.0148	0.0168	
177	0.0335	0.0160		**177**	0.0137	0.0105	
162	0.0275	0.0173		**162**	0.0122	0.0132	
Mean	**0.0233**	**0.0144**		**Mean**	**0.0111**	**0.0092**	
Total Survival							
GM12	102.50	84.92		**GM12**	5.16	13.75	
S54N	48.58	61.07		**S54N**	4.11	7.13	
114	47.78	89.67		**114**	5.66	15.52	
193	38.50	81.83		**193**	11.03	8.27	
UM9	109.83	95.82	0.107	**UM9**	12.16	16.28	0.040
176	22.80	59.67		**176**	1.61	5.47	
177	73.50	58.92		**177**	9.97	13.47	
162	42.33	81.33		**162**	10.10	9.64	
Mean	**60.73**	**76.65**		**Mean**	**7.48**	**11.190**	

3.3.5. Biofilm biomass

The biofilm biomass (after 6 and 24 hr periods) formed by each isolate was indirectly assessed by measuring the absorbance of CV stain described in section 3.2.5.1. All six UK and eight Nigerian MRSA isolates involved in this study were biofilm producers (as seen in Figure 3.11). The biofilm biomass for each isolate was seen to be higher at 24 hrs compared to the biomass at 6 hrs (Figure 3.11, Table 3.13). Despite this increase, UK isolate 16/11 had the least biofilm biomass after both 6 and 24 hr periods. After 6 hrs, UK isolate '3' had the highest CV stain absorbance at 0.228 while UK isolates 16/11 and Nasal had the least CV stain absorbances i.e. 0.174 (Figure 3.11, Table 3.13). After 24 hrs, UK isolates 16/11 and Shin were seen to have the least biofilm biomasses of all the isolates i.e. CV stain absorbance 0.37 and 0.42 respectively. UK isolates Nasal and 2 as well as Nigerian isolates UM9 and 177 have the highest biofilm biomasses as their CV stain absorbances were ~0.8. On the other hand, UK isolates Abdo and 3 as well as Nigerian isolates GM12, S54N, 114, 193 and 162 have biomasses at ~0.7; only Nigerian isolate 176 had biofilm biomass at CV absorbance ~0.6. Comparison of means of the biofilm biomasses (absorbance of CV stain) formed after 6 and 24 hrs showed that there were no significant differences (*P*-values >0.05) between the biomasses formed by UK and Nigerian MRSA (Tables 3.14 and 3.15). The Mann-Whitney U test also revealed that there were no significant differences between median values of the biofilm biomasses formed by the UK and Nigerian MRSA after both 6 and 14 hrs i.e. computed U values were > Ucrit (Tables 3.14 and 3.15).

Table 3.13: Crystal violet absorbance values for the biofilm biomass of MRSA isolates measured spectroscopically at 570 nm. Results are the average of three independent experiments. Values of $P < 0.05$ were accepted as significant based on the 1-tailed paired t-test. Difference between the medians of two data sets were considered significant if the computed U value was ≤ to Ucrit i.e. 10 (Mann-Whitney U test). Ucrit = 51 for 14 vs 14 samples.

Isolates	CV stain absorbance (570 nm)		
	After 6 hrs	After 24 hrs	
16/11	0.174	0.369	
Nasal	0.174	0.812	
Abdo	0.188	0.739	
Shin	0.186	0.420	
2	0.199	0.792	
3	0.228	0.654	
GM12	0.201	0.684	***P* value** 1.77E-09
S54N	0.193	0.719	
114	0.187	0.656	
193	0.190	0.712	
UM9	0.194	0.758	
176	0.184	0.636	**U value** 0
177	0.193	0.820	
162	0.207	0.737	
Mean	**0.193**	**0.679**	
Median	**0.1914**	**0.7155**	

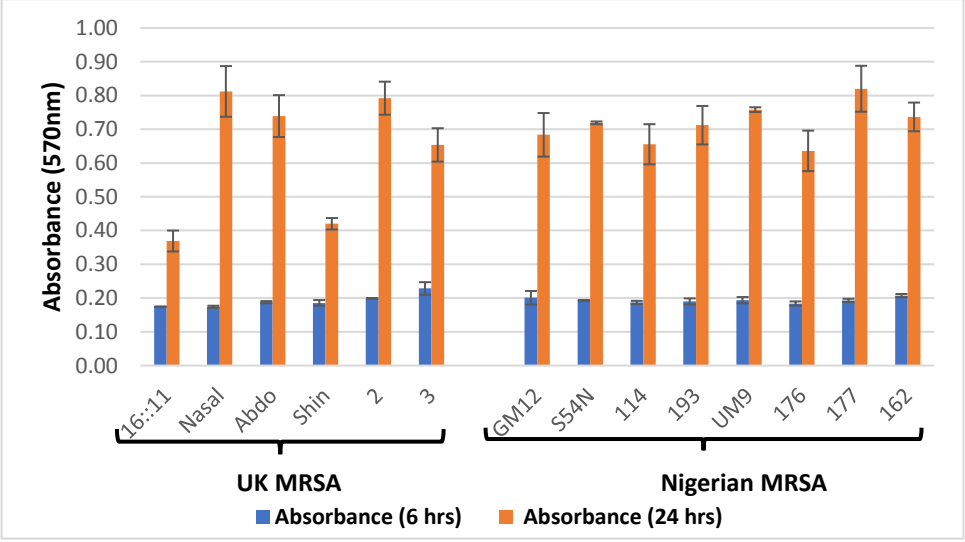


Figure 3.11: Crystal violet absorbance values for the biofilm biomass of MRSA isolates measured spectroscopically at 570 nm. Results are the average of three independent experiments. Error bars represent standard error.

Table 3.14: Crystal violet absorbance values for the biofilm biomass of MRSA isolates measured spectroscopically at 570 nm after 6 hrs. Results are the average of three independent experiments. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit i.e. 10 (Mann-Whitney U test).

UK Isolates	**CV stain absorbance (570 nm)**	**Nigerian isolates**	**CV stain absorbance (570 nm)**	
16/11	0.174	**GM12**	0.201	
Nasal	0.174	**S54N**	0.193	
Abdo	0.188	**114**	0.187	***P* value** 0.413
Shin	0.186	**193**	0.190	
2	0.199	**UM9**	0.194	
3	0.228	**176**	0.184	
		177	0.193	**U value** 17
		162	0.207	
Mean	**0.192**	**Mean**	**0.194**	
Median	**0.1867**	**Median**	**0.1930**	

Table 3.15: Crystal violet absorbance values for the biofilm biomass of MRSA isolates measured spectroscopically at 570 nm after 24 hrs. Results are the average of three independent experiments. Values of $P < 0.05$ were accepted as significant based on the unpaired one-tailed t test assuming unequal variance. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ to Ucrit (Mann-Whitney U test).

UK Isolates	**CV stain absorbance (570 nm)**	**Nigerian isolates**	**CV stain absorbance (570 nm)**	
16/11	0.369	**GM12**	0.684	
Nasal	0.812	**S54N**	0.719	
Abdo	0.739	**114**	0.656	***P* value** 0.171
Shin	0.420	**193**	0.712	
2	0.792	**UM9**	0.758	
3	0.654	**176**	0.636	
		177	0.820	**U value** 21
		162	0.737	
Mean	**0.631**	**Mean**	**0.715**	
Median	**0.6963**	**Median**	**0.7155**	

3.3. Discussion

Results from this study showed that the proportions of MRSA that invaded both KB and A549 cells were higher at MOI 10:1 than at MOI 100:1 (Figures 3.2 and 3.7, Tables 3.2 and 3.7). In other words, invasion of the cell lines with MRSA was more efficient (percentage of starting inoculum recovered as invaded/internalised at the end of the assay) at MOI 10:1 than at MOI 100:1. This finding is similar to results obtained in the study by Hu and Kopecko, 1999, where they saw that *Campylobacter jejuni* invaded human INT407 cells more efficiently at low MOIs of about 0.2 to 20 than at higher MOIs. It was proposed that the reduced infection efficiencies with very high MOIs, observed in the Hu and Kopecko, 1999 study with *C. jejuni*, are possible a consequence of the increased cytotoxic effects of *C. jejuni* on cultured epithelial cells (Backert and Hofreuter, 2013). This could possibly also be the case in this study as *S. aureus* expresses a wide range of virulence factors that cause specific tissue damage in the host (Lowy, 1998; Gordon and Lowy, 2008; Otto, 2010). Furthermore, in the Hu and Kopecko, 1999 study, it was found that although infection was less efficient at higher MOIs, the numbers of intracellular bacteria at the higher MOIs were higher compared to the numbers of intracellular bacteria following infection at lower MOIs. This finding is similar to the results obtained from this study which showed that although MRSA invasion of both KB and A549 cell lines was less efficient at MOI 100:1 (Figures 3.2 and 3.7, Tables 3.2 and 3.7), the number of intracellular/internalised bacteria after the invasion assays were higher at MOI 100:1 (UK MRSA [KB – 1,094,444 CFU; A549 – 810,000 CFU], Nigerian MRSA [KB – 1,105,417 CFU; A549 – 922,708 CFU) than at MOI 10:1 (UK MRSA [KB – 207,222 CFU; A549 – 144,444 CFU], Nigerian MRSA [KB – 232,500 CFU; A549 – 144,375 CFU]) (Tables E5B and E6B (Appendix E)).

At least one of either the fibronectin binding protein A or B genes (*fnbA* or *fnbB*), that encode the fibronectin binding proteins (FnBPs) A and B, is found in the vast majority of *S. aureus* strains (Brouilette *et al*., 2003). In line with this, results from Chapter two (Section 2.3.3) showed that all

six UK and eight Nigerian MRSA isolates involved in this study bear the *fnbA* gene (Figures 2.10 and D.9 (Appendix D), Table E.4 (Appendix E)). Results from the present study showed that except for intracellular survival in A549 cells at MOI 100:1 (*P* value = 0.0460; Table 3.9B), there is no significant difference between the mean proportions of UK and Nigerian MRSA that associated with, invaded or survived in both KB epithelial and A549 lung cell lines at both MOI 10:1 and 100:1 (Sections 3.3.1 – 3.3.2). The fact that the FnBPA has a higher affinity for fibronectin than FnBPB, and that the presence of *fnbA* alone in *S. aureus* is sufficient for adhesion and cell invasion (Josse *et al*., 2017), could explain why both the UK and Nigerian MRSA isolates interacted with both KB and A549 cells similarly regardless of the fact that majority of the Nigerian isolates and one UK isolate also bear the *fnbB* gene.

Staphylococcus aureus is known to cause infections in a variety of different tissues, however, colonisation of the host occurs with varying frequency at different sites of the body (Ridley *et al*., 2012). This variation may be due to differences in the capacity of *S. aureus* to adhere to and invade host cells present at those body sites (Ridley *et al*., 2012). Similar to findings from the study by Ridley *et al*., 2012, who compared the ability of cells from the oral cavity (H357 cell line), skin keratinocytes (UP cell line) and keratinocytes from the nasal cavity (Detroit 562 cell line) to adhere to and internalise *S. aureus*, and found that *S. aureus* adhered to and invaded all the tested cell lines with differing efficiencies, variations were also observed in the efficiencies at which MRSA isolates invaded the cell lines used in this study. As can be seen in Tables 3.11 and 3.12 (Sections 3.3.3 and 3.3.4 respectively), the mean proportions of UK and Nigerian MRSA that invaded KB cells were higher than the mean proportions that invaded A549 cells at both MOI 10:1 (KB [UK – 0.0207%; Nigerian – 0.0233%], A549 [UK – 0.014444%; Nigerian – 0.014438%]) and 100:1 (KB [UK – 0.0109%; Nigerian – 0.0111%], A549 [UK – 0.0081%; Nigerian – 0.0092%]). The difference was significant (*P* value = 0.033) when Nigerian MRSA invaded the cells at MOI 10:1 but not in other instances of invasion. In terms of association (adherence and internalisation), the

results showed that the mean proportions of both the UK and Nigerian MRSA isolates that associated with KB epithelial cells at MOI 100:1 (UK - 0.0254%; Nigerian – 0.0240%) were significantly higher levels than the proportions that associated with A549 cells (UK – 0.0178%; Nigerian – 0.0164%) (Tables 3.11 and 3.12). However, at MOI 10:1, the results also showed that the mean of proportions of UK MRSA isolates that associated with KB cells (0.0378%) was higher than the proportion that associated with A549 cells (0.0322%); this observed difference in association was not significant (Table 3.11). On the other hand, the percentage of Nigerian MRSA isolates that associated with KB cells was lower (0.0283%) than percentage that associated with A549 cells (0.0291%) (Table 3.12). Additionally, the results showed that the mean proportions of both the UK and Nigerian MRSA that survived in the A549 cells at MOIs 10:1 (UK – 62.85%; Nigerian – 76.65%) and 100:1 (UK – 7.49%; Nigerian – 11.19%) were higher compared with the mean proportions of survival in the KB cells MOIs 10:1 (UK – 47.22%; Nigerian – 60.72%) and 100:1 (UK – 5.10%; Nigerian – 7.48%) (Sections 3.3.3 and 3.3.4; Tables 3.11 and 3.12). These findings emphasise the fact that the host cell type plays an important role in the outcome of interaction between MRSA and mammalian cells.

In addition to the Ridley *et al*., 2012 study, other *in vitro* studies of invasion of host cells by *S. aureus* have also previously reported levels of invasion that vary widely between cell lines of different tissue origins including: bovine mammary-epithelial cells (Dziewanowska *et al*., 2000), kidney cells (Agerer *et al*., 2003), fibroblasts (Hussain *et al*., 2008), and human epithelial cell lines including HaCat keratinocyte (Mempel *et al*., 1998), the H357 oral squamous carcinoma and UP human keratinocyte cell lines (Kintarak *et al*., 2004). However, as proposed by Ridley *et al*., 2012, it is possible that the observed differences in adherence to and invasion of the host cells (i.e. KB and A549 cells in this study) is due to variations in host cell features, which would include the availability of cell-surface binding sites involved, in particular the integrin $\alpha_5\beta_1$. This α5β1 integrin, which is required for the invasion of host cells by *S. aureus*, is ubiquitously expressed on

human cells (Ridley *et al*., 2010). Lower total numbers of $\alpha_5\beta_1$ heterodimers, polarisation of $\alpha_5\beta_1$ so that it is relatively hidden, or $\alpha_5\beta_1$ heterodimers being relatively 'inactive' in terms of interaction with fibronectin, may all affect availability of $\alpha_5\beta_1$ (Ridley *et al*., 2012). Furthermore, these findings also suggest the possibility that the studied MRSA isolates have a specific tropism for skin (KB) rather than lung (A549) cells. Tropism refers to the ability of a given pathogen to infect a specific location, which could be an organ, set of organs or tissues (McCall *et al*., 2016). While some pathogens are broadly tropic, infecting all or most organs, others are restricted to a given tissue or even to certain tissue niches (McCall *et al*., 2016). Further supporting this possibility are the sites of isolation of the MRSA. Majority of the six UK and eight Nigerian MRSA isolates (83.33% and 62.50% respectively) were obtained from wounds (Table 2.1, Section 2.2.1). UK isolate Nasal was isolated from the nares of an individual, Nigerian isolate 193 was isolated from a high vaginal swab while Nigerian isolates 176 and 162 were obtained from blood samples (Table 2.1).

In order to determine if there were skews (i.e. unevenness) between the levels of interaction of UK and Nigerian MRSA with KB and A549 cells, medians of the levels of interaction (association, invasion and survival) of UK and Nigerian MRSA with KB and A549 cells were determined and compared using the Mann-Whitney U test. Differences between the medians indicates that the values in the two data sets (the calculated levels of interaction of UK MRSA and the calculated levels of interaction Nigerian MRSA with the cell lines) are not similar; either the values in one set are higher or lower than those in the othera set of data. Similar to results obtained from comparison of means using the t test, where it was seen that there were no significant differences in the means of the proportions of UK and Nigerian MRSA that interacted with both KB and A549 cells (P values > 0.05 in most instances of association, invasion and survival), results obtained from the Mann-Whitney U test also showed that there were no significant differences between the medians of the proportions of UK and Nigerian MRSA that interacted with (associated with,

invaded and survived in) both KB and A549 cells in most instances (Sections 3.3.1 – 3.3.2). This lack of significance in the medians of the data sets indicates that there is no skew in favour either the UK or Nigerian MRSA. In the first exception, although there was no significant difference between the means, it was seen that the median of the proportions of Nigerian MRSA that survived intracellularly in KB cells at MOI 100:1 was significantly higher than the median of the proportions of UK MRSA that survived intracellularly in KB cells at the same MOI (Section 3.3.1.3.2, Table 3.4B). The second exception was when it was seen that the mean proportion of Nigerian MRSA that intracellularly survived in A549 cells was significantly higher than that of UK MRSA i.e. *P* value = 0.0460; even though there was no significant difference in medians (Section 3.3.2.3.2, Table 3.9B).

The proportions of intracellular survival of Nigerian MRSA in A549 cells at MOI 100:1 ranged from 2.00% - 11.22% while the range was 5.33% - 14.83% for UK MRSA (Table 3.9B). Furthermore, proportions of Nigerian isolates GM12, 114, UM9 and 177 that survived intracellularly (13.50%, 14.83%, 13.50% and 13.00% respectively) were higher than the proportion of UK isolate 'Shin', which was the most efficient at surviving intracellularly (11.33%) among the UK isolates (Table 3.9B). The fact that there was no significance in the difference of the median values despite significant difference between the mean values (Section 3.3.2.3.2, Table 3.9B) indicates that although higher proportions of intracellular survival (i.e. higher efficiencies) were observed among the Nigerian MRSA, the proportions (i.e. data) were not skewed in favour of Nigerian MRSA.

A look at the proportions of both UK and Nigerian MRSA that survived intracellularly showed that the range of values of the Nigerian MRSA was wider (1.38% - 11.67%) than that of the UK MRSA (2.07% - 8.17%) at MOI 100:1 (Table 3.4B). Moreover, proportions of four of the eight Nigerian MRSA (isolates 193, UM9, 177 and 162), that survived intracellularly in KB cells were higher (10.17%, 11.67%, 9.50% and 8.67% respectively) than the proportion of UK MRSA 'Shin'

that had the highest intracellular survival (i.e. 8.17%) among the UK isolates (Table 3.4B). This skew in the distribution of proportions is the basis for the differences in the significance of the mean (insignificant) and median (significant) values.

To understand the basis for the unevenness observed in the distribution of proportions, it is important to remember that after uptake into host cells, the fate of both the pathogen and the host cells depend on the individual *S. aureus* strain (Krut *et al*., 2003; Fraunholz and Sinha, 2012). Therefore, the higher intracellular surviving proportions observed among the Nigerian MRSA could be because they are better equipped (virulence factor-wise) than the UK MRSA at surviving in both KB and A549 cells (Tables 3.4B and 3.9B). A comparison of the presence of the genes for virulence factors known to facilitate attachment/internalisation to host cells (*fnbA* and *fnbB*) and pore formation (*hlα, hlb, hlδ, hlγ*, PSMα i.e. cytotolysins known to facilitate *S. aureus* escape from the phagolysosome into the cytoplasm) showed that genes for these toxins were present in Nigerian isolate '176', (Section 2.3.3, Tables 2.3 and 2.5) which was the least efficient at surviving intracellularly in KB and A549 cells at MOI 100:1 (1.38% and 5.33% respectively) (Table 3.4B). On the other hand, UK isolate 'Shin', which was most efficient at surviving intracellularly in KB and A549 cells at MOI 100:1 (8.17% and 11.33% respectively) (Table 3.4B), was seen to bear the *fnbA* and *fnbB* genes as well as genes for the pore forming toxins PSMα, *hla, hlb* and *hlδ* except that for *hlγ* (Section 2.3.3, Tables 2.3 and 2.5). In other words, though the results showed that Nigerian isolate '176' was equipped with more virulence that are known to facilitate intracellular survival, its level of intracellular survival in KB cells was lower than that of UK isolate 'Shin' which had fewer virulence factors. Another case is seen with UK isolates '16/11' and Nigerian isolate 'GM12'; except for the *entC* gene which was present in isolate '16/11' but absent in 'GM12', both isolates are identical in terms of the virulence factors checked for in this study (Table 2.3). Nevertheless, the proportion of isolate 'GM12', which survived intracellularly in KB cells at MOI 100:1 was higher (4.68%) than the proportion of UK isolate '16/11' (2.07%) (Table 3.4B),

which had the additional *entC* gene. Thus, though the virulence factor profiling (done in this study) of both of UK isolate '16/11' and Nigerian isolate 'GM12' showed that both isolates were identical in terms of virulence factors that are known to facilitate internalisation and intracellular survival, they exhibited varying levels of intracellular survival.

Further emphasising the possible effect that the genotype of an infecting pathogen can have on the outcome of an infection can be seen in the results obtained from the comparison between the interaction of UK MRSA with KB and A549 cells (Section 3.3.4; Tables 3.11) as well as the comparison between the interaction of Nigerian MRSA with KB and A549 cells (Section 3.3.5; Table 3.12). Firstly, of all the Nigerian MRSA, isolate 'UM9' was seen to be the least efficient at associating with both cell lines at MOI 10:1 (KB - 0.0132%; A549 – 0.0150%) and KB cells at MOI 100:1 (0.0130%) (Table 3.12). It also invaded KB and A549 cells the least at both MOI 10:1 (KB – 0.0077%; A549 – 0.0053%) and 100:1 (KB – 0.0036%; A549 – 0.0033%). Nevertheless, summation of results from survival in cell culture supernatant and intracellular survival assays (presented as total survival in Table 3.12), showed that this isolate 'UM9' had the highest level of survival in both KB and A549 cells at both MOI 10:1 (KB - 109.83%; A549 – 95.82%) and 100:1 (KB – 12.16%; A549 – 16.28%). A look at the virulence factor profile of this isolate 'UM9' showed that it was the only Nigerian isolate used in the interaction assays that did not bear the *fnbB* gene (Table 2.3).

The fibronectin binding proteins A and B have been demonstrated to be involved in not only adhesion to cells but also internalisation by cells (Shinji *et al*., 2011). Despite the fact that the FnBPA has a higher affinity for fibronectin than FnBPB, and the fact that the presence of *fnbA* alone in *S. aureus* is sufficient for adhesion and cell invasion (Josse *et al*., 2017), Shinji *et al*., 2011 have demonstrated that FnBPA and FnBPB work together for the establishment of infection. There is therefore a possibility that among the Nigerian MRSA, the absence of the *fnbB* gene in 'UM9' (despite the presence of both *fnbA* and *fnbB* in the other Nigerian MRSA involved in the

interaction assays) was responsible for ‘UM9’ being the least efficient at associating with and invading the cell lines, but the most efficient at surviving in the cells. When the virulence factor profile of this Nigerian isolate was compared with those of the UK MRSA isolates, it was seen that five (83.33% i.e. isolates Nasal, Abdo, Shin, 2 and 3) of the six UK isolates did not also bear the *fnbB* gene (Table 2.3). Yet results from the interaction assays showed that UK isolates, lacking the *fnbB* gene, were the most efficient at associating with KB and A549 cells at MOI 100:1 (isolates Abdo [0.0355%] and Shin [0.0227%] respectively) and also invading KB and A549 cells at MOI 10:1 (isolates Abdo [0.0385%] and Shin [0.0225%] respectively) (Table 3.11). Furthermore, UK isolates ‘3’ and Abdo were the least efficient at surviving in KB and A549 cells, respectively, at MOI 10:1 (isolates 3 [6.82%] and Abdo [7.43%] respectively). Therefore, the presence or absence of the *fnbB* gene cannot be conclusively linked to the efficiency or otherwise of the interaction process. Therefore, although difference in expression of virulence factors may be responsible for the significant difference that was observed in the mean, median and the range of proportions of the UK and Nigerian isolates that survived in KB and A549 cells at MOI 100:1, it cannot be conclusively inferred from the virulence factor profiling done in this study.

A microtitre plate assay was used to compare the ability of the MRSA isolates involved in this study to form biofilms on the polystyrene surface of a 24-well plate. All the isolates involved in this study were seen to be biofilm formers. However, no significant differences were observed in the means and medians of the biofilm biomasses formed by UK and Nigerian MRSA after both 6 and 24 hrs (Tables 3.14 and 3.15). A comparison of biofilm biomass after 6 hrs (Section 3.3.5) and association of the MRSA isolates with KB cells (Section 3.3.1.1) showed that although UK isolate ‘3’ had the highest biomass (CV absorbance 0.228), it was not the isolate that had the highest level of association with KB cells at both MOIs 10:1 and 100:1. Rather, UK isolates 16/11 and Abdo associated with KB cells at the highest levels at MOIs 10:1 and 100:1 respectively (i.e. 0.0575% and 0.0355% respectively) (Figure 3.1, Table 3.1). Furthermore, it was Nigerian isolate 177 that

associated with A549 cells at the highest level at both MOIs 10:1 and 100:1 i.e. 0.0452% and 0.0268% respectively (Section 3.3.2.1, Table 3.6, Figure 3.6) not UK isolate 3 that has the highest biomass after 6 hrs (Figure 3.11, Table 3.13).

A comparison of biofilm formation after 24 hrs (Section 3.3.5) and association of the MRSA isolates with KB cells (Section 3.3.1.1) showed that although UK isolate 16/11 had the least biofilm biomass i.e. CV stain absorbance 0.369 (Figure 3.11, Table 3.13), it associated with KB cells at the highest level at MOI 10:1 (i.e. 0.0575%; Figure 3.1A, Table 3.1A). When it came to A549 cells, Nigerian isolate 177 which had the highest biofilm biomass after 24 hrs, i.e. CV absorbance 0.820, associated with A549 cells at the highest level at both MOIs 10:1 and 100:1 i.e. 0.0452% and 0.0268% respectively (Figure 3.6, Table 3.6). Therefore, findings from this study show that a higher biofilm biomass does not translate to a higher level of association with host tissues.

Despite extensive use, one of the draw backs of the microtiter plate method, for quantification of the *in vitro* biofilm forming capability of bacteria, is that results cannot be extrapolated to *in vivo* scenarios with confidence i.e. *in vitro* biofilm phenotype on microtiter plates does not necessarily speak for the *in vivo* biofilm phenotype of a particular strain (Fernandez-Barat *et al*., 2018). In testing the hypothesis that MRSA strains with weak *in vitro* biofilm-producing abilities could have increased ability under *in vivo* settings, Fernandez-Barat *et al*., 2018 demonstrated that a weak biofilm producing MRSA strain significantly enhances its biofilm production within an ETT. They found that this increased ability was influenced by the ETT environment and not the systemic treatment used during intubation or the atmospheric conditions used for bacterial growth. Therefore, although results from this study showed that all the MRSA isolates (both UK and Nigerian) used in this study have the capacity to produce biofilms *in vitro* (Section 3.3.5), this capacity may be enhanced or even diminished in an *in vivo* environment.

In conclusion, findings from this study showed that in addition to MOIs and genotype of the infecting strain, host tropism and host cell dependent properties played roles, in one way or another, in the interactions of MRSA (UK and Nigerian) with KB skin epithelial and A549 lung cell lines. Furthermore, differences in the virulence factor profiles of the isolates involved in this study could be responsible for the observed results. However, as only 13 of the vast array of virulence factors expressed by *S. aureus* was probed for in this study, no conclusion could be drawn as to which virulence factors were responsible for the findings. Thus, differences in behavioural patterns of UK and Nigerian isolates could not be firmly established based on the findings in this chapter.

Chapter Four

Cytotoxic effects of MRSA on Mammalian Cell Lines

4.1. Introduction

Staphylococcus aureus was once considered a non-invasive extracellular pathogen that damages host cells after adhering to the extracellular matrix (Alexander *et al*., 2003; Flannagan *et al*., 2016). However, it is now firmly established that *S. aureus* can invade various types of host cells (Balwit *et al*., 1994; Bayles *et al*., 1998; Bantel *et al*., 2001; Cardas *et al*., 2012; Flannagan *et al*., 2016). Different post-invasion events are possible following *S. aureus* invasion of host cells. These events can be attributed to the cumulative action of different bacterial components rather than to a single virulence factor (Strobel *et al*., 2016). Furthermore, the susceptibility of host cells to virulence factors as well as host cell gene expression, also play roles in the outcome following *S. aureus* invasion (Fraunholtz and Sinha, 2012).

The infecting strain could persist (as small colony variants [SCVs]) within morphologically intact host cells, (Strobel *et al*., 2016; Rollin *et al*., 2017). These naturally occurring variants gain a survival advantage by their ability to persist within eukaryotic cells, which protects them from host defences and antibiotics (Garcia *et al*., 2013). SCVs are characterised by non-pigmented, non-haemolytic colonies ~10 times smaller than those of the normal phenotype (Garcia *et al*., 2013). This tiny size is often due to auxotrophy for distinct growth factors such as menadione, haemin and/or thymidine (Garcia *et al*., 2013). Due of their very slow growth, populations of SCVs can be rapidly overgrown by any mutant bacteria in the population that reverts the original mutation, or that acquires a second-site intragenic suppressor mutation restoring activity to the mutated gene (Brandis *et al*., 2017). Furthermore, due to the fact that they grow slowly, they are more resistant to antibiotics (Fraunholtz and Sinha, 2012). An important part of the ability of *S. aureus* SCVs to persist intracellularly is associated with the quiescent metabolic state (Proctor *et al*., 2014). SCVs are generally found to be mutants in the accessory gene regulator locus (*agr*), thus failing to produce a variety of quorum sensing-controlled virulence factors (Fraunholtz and Sinha, 2012). *Staphylococcus aureus* SCVs thus produce fewer lytic enzymes, thereby allowing them to persist

within the host cells (Proctor *et al*., 2006; Proctor *et al*., 2014; Tuchscherr *et al*., 2010). On the other hand, they show increased expression of adhesins that facilitate host cell uptake (Proctor *et al*., 2006; Tuchscherr *et al*., 2010; Proctor *et al*., 2014). Moreover, the failure of *S. aureus* SCVs to stimulate host cells to produce hypoxia-inducible factor, which would normally alert the host to the presence of intracellular pathogens, is also important for promoting persistence (Proctor *et al*., 2014). Furthermore, SCVs display a thick cell wall and an up-regulation of alternative sigma-factor σB, which enables *S. aureus* to cope with a variety of environmental stressors (Fraunholtz and Sinha, 2012). Infection could also result in rapid lysis of the host cell which comes about because of the secretion of toxins and other pro-inflammatory factors that induce strong inflammatory and cytotoxic effects (Rollin *et al*., 2017).

Though non-professional phagocytic cells (NPPCs) don't have the bactericidal endowments of professional phagocytes, they exhibit various mechanisms of disposing with invading pathogens (Loffler *et al*., 2014). One of such mechanisms is autophagy, an ubiquitous process responsible for eliminating harmful protein aggregates, intracellular pathogens, and unnecessary proteins by the lysosomes (Ghosh and Pattison, 2018). Autophagy is used to combat intracellular pathogens in case of an inefficient phagosomal degradation pathway (Schnaith *et al*., 2007). In addition to the principal function of removing unwanted or damaged cellular constituents, it also functions principally to supply the cell with substrates for energy generation in times of nutrient deprivation (Wang *et al*., 2010).

Autophagy can be categorised into three groups, i.e. macroautophagy, microautophagy and chaperon-mediated autophagy (CMA) (Yoshii and Muzishima, 2017; Ghosh and Pattison, 2018). When macroautophagy is induced, an isolation membrane encloses a portion of cytoplasm, forming a characteristic double-membraned organelle termed the autophagosome (Yoshii and Muzishima, 2017). Damaged cytosolic components are removed and recycled in double-membrane vacuoles, called autophagosomes, which present microtubule-associated protein 1 light

chain 3 (LC3) associated to phosphatidylethanolamine (LC3-II). (O'Keeffe *et al*., 2015; Darisipudi *et al*., 2018). The autophagosomes then fuse with the lysosomes to form autophagolysosomes, the contents of which are then degraded by lysosomal enzymes (Yoshii and Muzishima, 2017; Ghosh and Pattison, 2018). This process of autophagosome formation and eventual degradation is termed autophagic flux (O'Keeffe *et al*., 2015). In microautophagy, the lysosome or vacuole itself engulfs a portion of cytoplasm either by invagination of the lysosomal membrane or protrusion of the membrane to surround the cytosol or organelles (Yoshii and Muzishima, 2017). However, in CMA, proteins are unfolded and directly translocated into lysosomes dependently on the Lys-Phe-Glu-Arg-Gln (KFERQ)-like motif of the substrate, and on cytosolic heat shock-cognate chaperone of 70 kDa (HSC70), lysosome-associated membrane protein type-2A (LAMP2A) and lysosome resident HSC70 (Yoshii and Muzishima, 2017; Ghosh and Pattison, 2018). Phagocytosed *S. aureus* are, however, able to evade lysosomal killing by disintegration of the organelle membrane (phagosome, autophagosome) in order to translocate into the host cytoplasm (Fraunholtz and Sinha, 2012; Loffler *et al*., 2014); this ability is linked to host cell death (Menzies and Kourteva, 1998; Loffler *et al*., 2014, Ridley *et al*., 2017).

Generally, the intracellular destination of bacteria is the cytosol or a membrane-bound vacuole; however, regardless of their niche, intracellular pathogens must eventually escape the confines of a host cell (Hybiske and Stephens, 2008). Exit of human pathogens from host cells is typically linked to tissue inflammation, organ dysfunction, and host-to-host transmission, thereby significantly contributing to disease burden and the epidemiology of infectious diseases (Flieger *et al*., 2018). Intracellular pathogens have evolved several pathways to escape the confines of a host cell, i.e. programmed cell death including the non-lytic apoptosis and the lytic necroptosis and pyroptosis pathways; the active breaching of host cell-derived membranes such as the endosomal, the vacuolar and/or the host cell plasma membrane; and the induced membrane-dependent exit without host cell lysis, e.g. via actin-based protrusions, extrusions, budding,

exocytosis, expulsion or ejection (Flieger *et al*., 2018). The pathways follow a spatially and temporally defined coordinated process and involve the interaction of pathogen- and host-derived effector molecules (Flieger *et al*., 2018). Depending on life-cycle stage, environmental factors and/or host cell type, majority of intracellular pathogens can utilise more than one of these pathways (Flieger *et al*., 2018).

Successful intracellular pathogens must suppress programmed cell death signals during the replicative or latent phase, nevertheless, they are also able to induce these signals to promote egress (i.e. exit from host cell) and dissemination (Traven and Naderer, 2014). Apoptosis is characterised by typical morphological changes, including the disintegration of the apoptotic cell into condensed apoptotic bodies, which are subsequently taken up by phagocytes (Flieger *et al*., 2018). Via uptake of these apoptotic bodies, viable pathogens could be transferred to phagocytic cells in a non-inflammatory context (Flieger *et al*., 2018). Necroptosis, a mode of programmed cell death negatively regulated by caspases and dependent on the kinase activity of receptor-interacting proteins, is characterised by a rapid membrane breakdown which shows morphological features similar to necrosis (Negroni *et al*., 2015). Furthermore, programmed cell death by pyroptosis, is a caspase-dependent form of necrotic cell death characterised by formation of pores in the plasma membrane and release of pro-inflammatory cytokines (Yuan *et al*., 2016). Though both pyroptosis and necroptosis trigger potent antimicrobial immune responses, these types of lytic host cell death also result in rapid release of intracellular pathogens, enabling dissemination and replication in rich extracellular niches (Traven and Naderer, 2014). Active host cell destruction describes an exit process, during which microbial molecules (such as proteases, phospholipases, and pore-forming proteins) penetrate or perforate membranes, such as the host cell plasma membrane or the membrane of the vacuolar compartment, consequently destroying host cell and compartment, respectively (Flieger *et al*., 2018). Propulsive forces due to actin polymerisation, which generates protrusions at the plasma membrane, also facilitate escape and spread of cytosolic microbes

(Negroni *et al*., 2015). These protrusions are engulfed by neighbouring cells, facilitating transfer of intracellular pathogens, such as Shigella, without host cell death (Negroni *et al*., 2015).

Staphylococcus aureus expresses a wide array of virulence factors, including multiple toxins, which interfere with host immune function (Das *et al*., 2016). These toxins elicit cytotoxicity toward a variety of cells ranging from epithelial cells to leukocytes, and their secretion is associated with lethality in some disease models (Das *et al*., 2016). Virtually all *S. aureus* toxins are under the control of the pivotal virulence regulator - accessory gene regulator (*agr*) (Cheung *et al*., 2011) a major regulator of *S. aureus* cytotoxicity and haemolysis (Das *et al*., 2016). The *agr*-system is known to upregulate toxins and a wide variety of virulence determinants, e.g. exoenzymes, and to downregulate expression of surface binding proteins at a certain level of cell density by a process called quorum sensing (Cheung *et al*., 2011).

Pro-inflammatory and cytotoxic virulence factors regulated by the accessory gene regulator (*agr*) system, play a significant role in the inflammatory and cytotoxic effects bacteria on host cell (Loffler *et al*., 2014). Among the *agr*-regulated factors, the pore forming α-toxin is known to be important in the induction of inflammation and cell death when expressed within the intracellular location in diverse cell types (Loffler *et al*., 2014). This α-toxin is able to bind to the host cell plasma membrane via A disintegrin and metalloprotease 10 (ADAM10) receptor on the host cell (Fraunholz and Sinha, 2012; Thay *et al*., 2013; Loffler *et al*., 2014). Following binding, α-toxin mediated cytotoxicity is achieved by destruction of the plasma membrane, leak of cellular ions or via delivery of toxic compounds through pores (Prince *et al*., 2012). The α-toxin mediates *S. aureus* phagosomal escape (Schnaith *et al*., 2007; Fraunholz and Sinha, 2012); it also participates in the activation of the autophagic pathway and facilitate autophagosomal escape (Loffler *et al*., 2014). However, though the α-toxin plays a crucial role in bacterial escape from phagosomes, it's been demonstrated that the α-toxin alone is not sufficient to mediate this escape (Giese *et al*., 2009). Giese *et al*., 2011 demonstrated that synergistic activity between the staphylococcal δ-toxin

and the sphingomyelinase β-toxin enabled the phagosomal escape of staphylococci in human epithelial and endothelial cells.

The α-toxin is produced by most *S. aureus* strains; depending on the target cell and dosage of the toxin, secreted α-toxin provokes a series of events (Thay *et al*., 2013). For instance, it activates and kills mainly mononuclear cells via apoptotic pathways (Loffler *et al*., 2014). Also, at sub-lytic concentrations, it predominantly induces apoptosis and/or inflammatory response in target cells while at high concentrations it mainly produces necrosis (Prince *et al*., 2012; Thay *et al*., 2013). In many cases, *S. aureus*-induced apoptosis has been proposed to require prior internalisation of staphylococci (Loffler *et al*., 2005) Time also determines the effect of α-toxin on the target cell; in epithelial cells for instance, a transient phase of apoptosis is followed by cellular necrosis (Prince *et al*., 2012). While apoptosis is defined as an active, programmed process of autonomous cellular dismantling that avoids eliciting an inflammatory response, necrosis is characterised as an accidental cell death resulting from environmental stimuli that elicits uncontrolled release of inflammatory cellular contents (Fink and Cookson, 2005).

Findings from studies like those by Denis *et al*., 2005, Schleucher *et al*., 2008, Beilouny *et al*., 2008, Zanger *et al*., 2012, reported cases of *S. aureus*-related infections in travellers returning from Africa. These findings have suggested that African *S. aureus* might have a different genetic background and might be more virulent than isolates from Europe (Schaumburg *et al*., 2014). The aim of this study was therefore to determine if Nigerian MRSA are more virulent than their UK counterparts. To do this, the cytotoxic effects of six UK and eight Nigerian MRSA isolates on mammalian cell lines (KB skin epithelial and A549 lung cell lines) were assessed. Toxic effects of the cell-free conditioned medium (medium that has previously supported bacterial growth and thus contains numerous secreted metabolites and signalling molecules endogenous to the particular strain of bacteria) of the UK and Nigerian MRSA on mammalian cells were assessed in addition to the toxic effects of heat-killed and live MRSA. In addition to these, the contribution of apoptosis

and necrosis to the observed cytotoxic effects of live MRSA on the mammalian cell lines was assessed in flow cytometry assays.

4.2. Materials and Methods

All chemicals were purchased from Sigma, unless otherwise stated.

4.2.1. Cell lines and cell culture

Human KB epithelial and A549 lung cell lines (carcinoma cells) were used in this study and were cultured, maintained, passaged and seeded in 24-well plates as described in Chapter Three (sections 3.2.1, 3.2.2 and 3.2.3).

4.2.2. Bacterial strains and culture

All six UK MRSA isolates (16/11, Nasal, Abdo, Shin, 2, 3) and eight Nigerian MRSA isolates (GM12, S54N, 114, 193, UM9, 176, 177, 162), also used for the interaction assays (Chapter three) were used in the cytotoxicity and flow cytometry studies described in this chapter. These isolates were grown and maintained as described in Chapter two (2.2.1). Optical densities of bacterial broth cultures were measured in a spectrophotometer as previously described in Chapter 2 i.e. OD_{595} 0.22. To prepare heat-killed (HK) bacteria, 3 ml of bacterial culture (OD_{595} 0.22) were put in an 80^{o}C water bath for 30 mins. Absence of growth on CLED agar confirmed killing.

4.2.3. Preparation of MRSA Conditioned Medium (CM)

One or two single colonies of MRSA were inoculated in LB broth and incubated for 6 hrs at 37^{o}C under aerobic conditions. 10 µl (~10^6 CFU) or 100 µl (~10^7 CFU) from the broth culture of each MRSA isolate (OD_{595} 0.22) was then inoculated in 5 ml of EMEM (for KB cell line) and DMEM (for A549 cell line) and further incubated overnight under aerobic conditions at 37^{o}C. Following incubation, bacteria were removed from the medium by centrifugation at 5000 rpm for 5 mins. The supernatant (CM) was then filtered using 0.2 µM syringe filters (Sarstedt, Numbrecht, Germany) to remove residual bacteria.

4.2.4. Cytotoxicity assays

The cytotoxic effects of two infectious doses (IDs) i.e. ~10^6 and ~10^7 CFU of MRSA on mammalian cells were evaluated in this study. Bacteria (live and heat killed) were added to the mammalian cells prepared in 4.2.1. at the rate of ~10^6 or ~10^7 CFU per well which correspond to MOIs 10:1 and 100:1 respectively. Bacterial conditioned media were prepared using ~10^6 CFU of bacteria per 5 ml of medium or ~10^7 CFU of bacteria per 5 ml of medium as a basis for comparison with the cytotoxic effects of ~10^6 CFU per well (MOI 10:1) or ~10^7 CFU per well (MOI 100:1) on cell lines. Control wells free of live bacteria, heat-killed bacteria or bacterial CM were set up. Controls as described in section 3.2.4. (Chapter three) were also set up for this experiment.

4.2.4.1. Infection of mammalian cell lines with live MRSA

Survival assays were performed as described in chapter three (section 3.2.4.4) except that after the elimination of un-internalised bacteria (with Vancomycin) and the subsequent addition of medium to each well, plates were incubated for both 6 and 24 hrs at 37ºC (in humidified 95% air, 5% CO_2).

KB and A549 mammalian cell lines were cultured and grown in 24-well plates in appropriate medium and incubated until 80-100% confluence was attained (~10^5 cells per well for both KB and A549 lung cell lines). Growth medium from each confluent well was refreshed thereafter and each well infected with live MRSA from an overnight broth culture at the rate of ~10^6 or ~10^7 CFU per well (corresponding to MOIs 10:1 and 100:1 respectively). Plates were then incubated at 37ºC (in humidified 95% air, 5% CO_2) for 40 mins, following which supernatants were discarded and each well washed with PBS to remove non-adherent bacteria. Vancomycin was added to each well at a final concentration of 100 µg/ml and incubated at 37ºC (in humidified 95% air, 5% CO_2) for 45 mins to kill off any un-internalised bacteria. Supernatant from each well was then discarded and the wells washed again with PBS. Following the killing of un-internalised bacteria supernatant from each well was discarded and the wells washed again with PBS. Thereafter, 500 µl of medium

appropriate for each cell line were added to each well, and plates were then incubated for both 6 and 24 hrs at 37^{o}C (in humidified 95% air, 5% CO_2).

4.2.4.2. Assays to assess the cytotoxic effects of heat-killed MRSA and MRSA CM on mammalian cell lines

Confluent wells of mammalian cells from which the growth medium had been removed were washed once with PBS**.** Following this, heat-killed (HK) bacteria (at the rate of ~10^6 CFU per well or ~10^7 CFU per well) and MRSA CM (from the ~10^6 CFU of bacteria per 5ml of medium or ~10^7 CFU of bacteria per 5ml of medium preparations) were added to each well as appropriate and incubated at 37^{o}C (in humidified 95% air, 5% CO_2) for 6 and 24 hrs.

4.2.5. Measurement of lactate dehydrogenase

Following the incubations described in section 4.2.4, the toxic effect of the different variables on cell lines were determined in a colorimetric assay which measures the activity of lactate dehydrogenase-LDH (this enzyme is released by cells with damaged plasma membranes and dead cells) in the supernatants using the Cytotoxicity Detection Kitplus; Roche. The LDH activity in the supernatants is determined by a coupled enzymatic reaction whereby the yellow tetrazolium salt INT in the dye solution (solution 2) is reduced to red formazan (dye). As cells get damaged or die off, the amount of LDH in the supernatants increase proportionally, therefore, the number of damaged or lysed cells directly correlates with the amount/colour of formazan formed in the assay.

Lysis buffer (5 μl) was added to some control wells to bring about release of a high amount of LDH from the cells; these were the high controls, while the low controls were just cells alone in growth medium. Fifty μl of supernatant were collected from each well and loaded in a fresh 96-well plate. Fifty μl of a reaction mixture (solution 1 + solution 2) were then added to all wells (sample and control) and incubated for 15 mins at room temperature. Next, the stop solution was

added to all wells to stop all reactions. Lastly, absorbance in each well was read at a wavelength of 595 nm in a FLUOstar Omega Multiplate Reader (BMG Labtech). Percentage cytotoxicity was calculated as follows:

$$\left(\%\ Cytotoxicity = \frac{[Sample\ value - \ Low\ control\ value]}{[High\ control\ value - \ Low\ control\ value]} \times 100\right)$$

4.2.6. Flow Cytometric analysis

To determine the distribution of apoptotic and necrotic cells following infection of mammalian cell lines with MRSA, mammalian cell lines were infected with MRSA as described in section 4.2.4.1. Thereafter, the cells were stained with the Fluorescein Isothiocyanate (FITC)_Annexin V (BioLegend) and Zombie NIR (BioLegend) dye. Cell-only control wells were also set up for this experiment.

Supernatants were removed from each well after the 24-hr incubation described in section 4.2.4.1 and stored in separate Eppendorf tubes. Each well was then washed with PBS and the wash liquid collected in the same tube as the supernatants. One hundred and fifty µl of accutase were then added to each well and incubated for 5 mins at 37^{o}C (in humidified 95% air, 5% CO_2). Thereafter, plates were taken out of the incubator and tapped gently to ensure total de-attachment of cells. Five hundred µl of medium with PBS from each Eppendorf tube were used to wash corresponding wells and the wash liquid collected back into the Eppendorf tubes. These cell suspensions were centrifuged at 1500 rpm/302 x g for 5 mins after which the cell pellets were washed twice with cell staining buffer (BioLegend) (200 µl of buffer and centrifugation at 1500 rpm/302 x g for 5 mins for each wash). The cell pellets in each Eppendorf tube, along with those of the cell only controls, were then re-suspended in 100 µl of cell binding buffer (BioLegend) plus 5 µl of FITC_Annexin V (BioLegend) and 1 µl of Zombie NIR (BioLegend).

Tubes were centrifuged at 1000 rpm/134 x g for 5 mins after 20 min incubation at room temperature in the dark. The supernatant from each tube was discarded and cells were fixed by adding 100 µl of 4% paraformaldehyde (PFA). This was followed by 10-30 mins incubation on ice. Four hundred µl of cell binding buffer were then added to each tube after which the cells were analysed by flow cytometry (The BD Accuri™ C6) with each run restricted to 10,000 moments.

4.2.7. Statistical analysis

Unless otherwise stated every experiment was repeated 3 times. Experimental data were analysed using a one-tailed unpaired t-test (two-sample unequal variance) for comparison between means. *P* values were considered statistically significant at a 95% confidence interval (i.e. $P < 0.05$). The Mann-Whitney U test was further used for comparison of medians; computed U values were compared to the critical value of U at a confidence interval of 95% / significance value of $P = 0.05$ (U critical = 10 for 6 vs 8 number of samples [One tailed Mann-Whitney U table for critical values: Appendix F]). Difference between the medians of two data sets were considered significant if the computed U value was less than or equal to the critical value (Ucrit).

4.3. Results

4.3.1. Cytotoxic effects of MRSA on mammalian cell lines

Unless otherwise stated, means were compared using one-tailed unpaired t-test while medians were compared using the Mann-Whitney U test.

4.3.1.1. Cytotoxic effects of MRSA on KB epithelial cell line

4.3.1.1.1. Cytotoxic effects at multiplicity of infection 10:1 six hours post-infection

Results obtained from this study showed that at MOI 10:1, the cytotoxic effects of live Nigerian MRSA on KB cells ranged from 0.36% - 8.24% six hrs post-infection (Table 4.1). The mean of these values (4.42%) was seen to be significantly higher (*P* value = 0.02817) than the mean of the cytotoxic effects of live UK MRSA (range from 1.08% - 3.70%, mean = 2.12) under the same conditions (Figure 4.1A, Table 4.1). A comparison of means however showed that the median of the cytotoxic effects of live Nigerian MRSA on KB cells (4.02%), was not significantly higher than that of UK MRSA (1.94%) i.e. calculated U value was higher than 10, which is the critical U value for the data sets (Table 4.1).

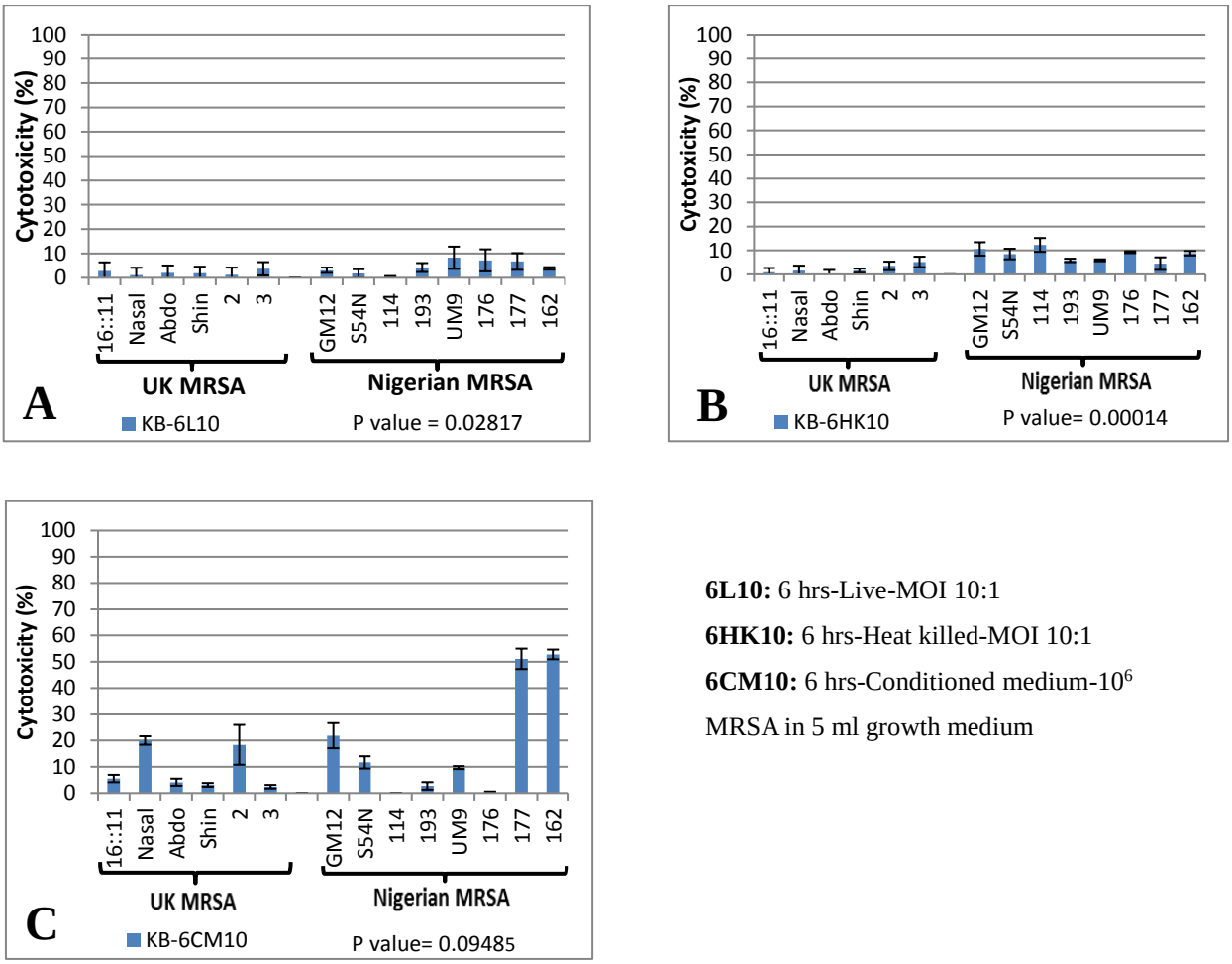
The mean of the cytotoxic effects of heat killed Nigerian MRSA at MOI 10:1 (range from 4.48% - 12.28%) was seen to be significantly greater than the mean cytotoxic effects of UK MRSA (range from 0.22% - 5.17%) on KB cells six hrs post-infection (Figure 4.1B, Table 4.1). Comparison of medians also showed that median of the cytotoxic effects of heat killed Nigerian MRSA on KB cells at MOI 10:1 (8.65%) was significantly higher (calculated U value [1] $\leq$ Ucrit [10]) than the median of cytotoxic effects of UK MRSA on KB cells (Table 4.1).

Results further showed that the mean cytotoxic effect of the CM of Nigerian MRSA (prepared by inoculating 10^6 CFU bacteria in 5 ml EMEM) on KB cells (range from 0.00% - 52.80%) was not significantly higher (*P* value = 0.09485) than the mean cytotoxic effects of the CM of UK MRSA

(prepared in the same way as those of their Nigerian counterparts) on KB cells (range from 2.06% - 18.38%) (Figure 4.1C. Table 4.1). Similarly, median of the cytotoxic effects of the CM of Nigerian MRSA (prepared by inoculating 10^6 CFU bacteria in 5 ml EMEM) on KB cells was also seen to be insignificantly higher (10.68%) than that of their UK counterparts (4.05%) i.e. Ucrit [10] < calculated U value [20] (Table 4.1).

Table 4.1: Cytotoxic effects of Live, Heat Killed, and Conditioned Media of MRSA at MOI 10:1 on KB epithelial cell line six hours post-infection. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on KB epithelial cell line at MOI 10:1 6 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	2.84	GM12	3.10	
Nasal	1.08	S54N	1.75	***P* value** 0.02817
Abdo	2.00	114	0.36	
Shin	1.88	193	4.21	
2	1.20	UM9	8.24	
3	3.70	176	7.17	**U value** 11
		177	6.69	
		162	3.83	
Mean	**2.12**	**Mean**	**4.42**	
Median	**1.94**	**Median**	**4.02**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	0.95	GM12	10.60	
Nasal	1.49	S54N	8.48	***P* value** 0.00014
Abdo	0.22	114	12.28	
Shin	1.52	193	5.81	
2	3.52	UM9	5.88	**U value** 1
3	5.17	176	9.25	
		177	4.48	
		162	8.81	
Mean	**2.14**	**Mean**	**8.20**	
Median	**1.50**	**Median**	**8.65**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	4.48	GM12	21.86	
Nasal	12.47	S54N	11.67	***P* value** 0.09485
Abdo	3.61	114	0.00	
Shin	2.06	193	2.73	
2	18.38	UM9	9.69	
3	2.06	176	0.29	**U value** 20
		177	51.12	
		162	52.80	
Mean	**7.18**	**Mean**	**18.77**	
Median	**4.05**	**Median**	**10.68**	



6L10: 6 hrs-Live-MOI 10:1

6HK10: 6 hrs-Heat killed-MOI 10:1

6CM10: 6 hrs-Conditioned medium-10^6 MRSA in 5 ml growth medium

Figure 4.1: Cytotoxic effects of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 10:1 on KB epithelial cell line six hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.1.2. Cytotoxic effects at multiplicity of infection 10:1 twenty-four hours post-infection

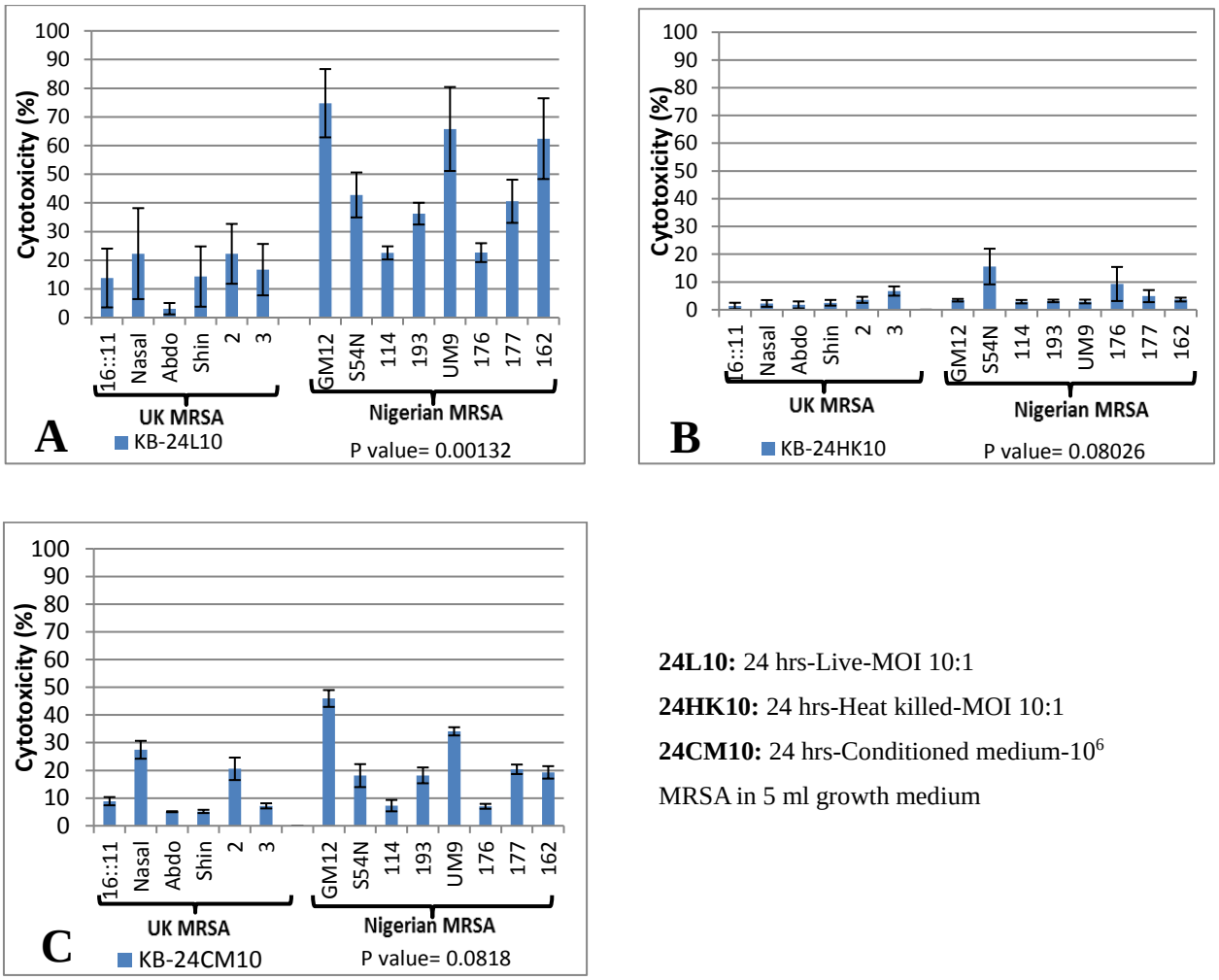
Twenty-four hours post-infection at MOI 10:1, UK isolate Abdo exerted the least cytotoxic effect (2.05%) on KB cells of all the live isolates (UK and Nigerian) (Table 4.2). On the other hand, it was Nigerian isolate GM12 that exerted the greatest cytotoxic effect on KB cells (74.78%) among all the isolates (Table 4.2). A comparison of the means of the cytotoxic effects of live MRSA isolates showed that the Nigerian isolates were significantly more toxic (45.79%) than their UK counterparts (15.24%) (Figure 4.2A, Table 4.2). A significant difference was also seen in the median of the cytotoxic effects of live UK MRSA (15.52%) and the median of the cytotoxic effects of live Nigerian MRSA (41.67%) on KB cells i.e. calculated U value [0] < Ucrit [10] (Table 4.2).

The range of the cytotoxic effects of heat killed UK MRSA was seen to be from 1.37% - 6.74%, while that of the heat killed Nigerian MRSA was seen to range from 2.90% - 15.56% (Table 4.2). However, even though the mean cytotoxic effect of the Nigerian isolates on the KB cells (5.73%) was higher than that of the UK isolates (3.03%), the difference was not significant (Figure 4.2B, Table 4.2). However, the median cytotoxic effect of the Nigerian isolates was seen to be significantly higher (3.55%) that that of the UK isolates (2.39%) i.e. calculated U value [10] $\leq$ Ucrit [10] (Table 4.2).

The range of the cytotoxic effects of the CM (prepared by inoculating 10^6 CFU bacteria in 5 ml EMEM) of Nigerian isolates was seen to be higher (7.03% - 45.95%) than that of their UK counterparts (5.07% - 27.44%) (Table 4.2). A comparison of medians showed that the median cytotoxic effect of the Nigerian isolates was significantly higher (18.74%) than that of the UK isolates (8.04%) i.e. calculated U value [7] < Ucrit [10] (Table 4.2). Regardless of this, the difference in the mean cytotoxic effect of the UK (12.39%) and Nigerian (21.21%) isolates was not significant (Figure 4.2C, Table 4.2).

Table 4.2: Cytotoxic effects of Live, Heat Killed, and Conditioned Media of MRSA at MOI 10:1 on KB epithelial cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was ≤ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on KB epithelial cells at MOI 10:1 24 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	13.80	**GM12**	74.78	
Nasal	22.31	**S54N**	42.78	***P* value** 0.00132
Abdo	2.05	**114**	22.56	
Shin	14.31	**193**	36.26	
2	22.24	**UM9**	65.78	
3	16.73	**176**	22.66	**U value** 0
		177	40.57	
		162	62.41	
Mean	**15.24**	**Mean**	**45.79**	
Median	**15.52**	**Median**	**41.67**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	1.37	**GM12**	3.43	
Nasal	2.28	**S54N**	15.56	***P* value** 0.08026
Abdo	1.74	**114**	2.90	
Shin	2.51	**193**	3.17	
2	3.57	**UM9**	2.96	
3	6.74	**176**	9.28	**U value** 10
		177	4.90	
		162	3.66	
Mean	**3.03**	**Mean**	**5.73**	
Median	**2.39**	**Median**	**3.55**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	8.88	**GM12**	45.95	
Nasal	27.44	**S54N**	18.11	***P* value** 0.0818
Abdo	5.07	**114**	7.27	
Shin	5.18	**193**	18.20	
2	20.57	**UM9**	34.09	
3	7.19	**176**	7.03	**U value** 7
		177	20.39	
		162	19.28	
Mean	**12.39**	**Mean**	**21.21**	
Median	**8.04**	**Median**	**18.74**	



24L10: 24 hrs-Live-MOI 10:1

24HK10: 24 hrs-Heat killed-MOI 10:1

24CM10: 24 hrs-Conditioned medium-10^6 MRSA in 5 ml growth medium

Figure 4.2: Cytotoxic effect of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 10:1 on KB epithelial cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.1.3. Cytotoxic effects at multiplicity of infection 100:1 six hours post-infection

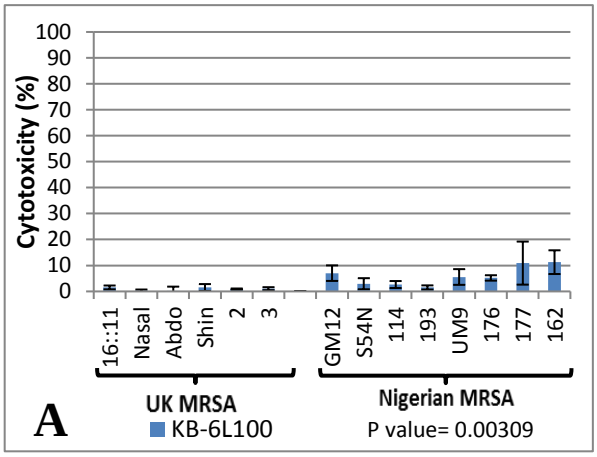
At MOI 100:1, the cytotoxic effects of live UK MRSA on KB cells (0.22% - 1.52%) was seen to be lower than that of the Nigerian MRSA (1.51% - 11.27%) 24 hrs after infection (Table 4.3). The observed differences in the mean cytotoxic effects of the live UK and Nigerian MRSA, 0.90% and 4.15% respectively, was seen to be significant (Figure 4.3A, Table 4.3). A significant difference was also seen in the median cytotoxic effects of live UK and Nigerian MRSA (0.96% and 5.37% respectively) as calculated U value [1] was < Ucrit [10] (Table 4.3).

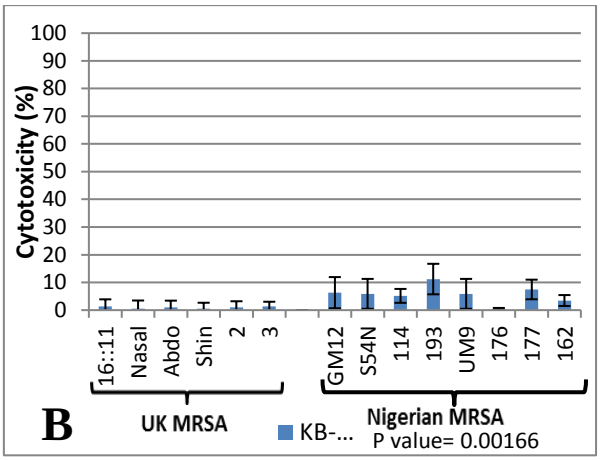
With regards the cytotoxic effects of heat killed MRSA on KB cells at MOI 100:1, the Nigerian MRSA were seen to be more toxic than the UK MRSA as the mean toxicities were calculated to be 5.72% and 0.98% respectively (Table 4.3); this observed difference was also significant (Figure 4.3B, Table 4.3). The median cytotoxic effect of heat killed Nigerian MRSA (5.89%) was also seen to be significantly higher that of UK MRSA (1.04%) on KB cells i.e. calculated U value [7] < Ucrit [10] (Table 4.3).

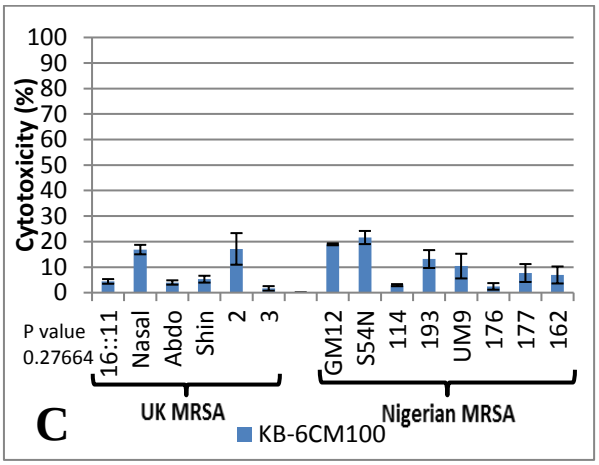
Among the UK isolates, the CM (prepared by inoculating 10^7 CFU bacteria in 5 ml EMEM) of isolate 3 exerted the least toxic effect on KB cells while isolate 2 exerted the greatest effect. Among the Nigerian isolates, the CM of isolate 176 exerted the least toxic effect on KB cells (2.45%) while that of isolate S54N exerted the most effect (21.60%). A comparison of the mean toxic effects of the UK and Nigerian CM on KB cells (8.22% and 10.52% respectively) showed that there was no significant difference (Figure 4.3C, Table 4.3). A further comparison of medians also showed that no significant difference exists between the median cytotoxic effects of UK CM (4.84%) and Nigerian CM (9.05%) on KB cells as the calculated U value (30) was not $\leq$ the critical U value (10) (Table 4.3).

Table 4.3: Cytotoxic effects of Live, Heat Killed and Conditioned Media of MRSA at MOI 100:1 on KB epithelial cell line 6 hours post-infection. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on KB epithelial cells at MOI 100:1 6 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	1.52	**GM12**	7.03	
Nasal	0.28	**S54N**	2.95	
Abdo	0.22	**114**	2.65	***P* value** 0.00309
Shin	1.49	**193**	1.51	
2	0.88	**UM9**	5.55	
3	1.04	**176**	5.19	**U value** 1
		177	10.91	
		162	11.27	
Mean	**0.90**	**Mean**	**4.15**	
Median	**0.96**	**Median**	**5.37**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	1.34	**GM12**	6.31	
Nasal	0.55	**S54N**	5.89	
Abdo	1.05	**114**	5.15	***P* value** 0.00166
Shin	0.50	**193**	11.22	
2	1.03	**UM9**	5.89	
3	1.39	**176**	0.40	**U value** 7
		177	7.47	
		162	3.48	
Mean	**0.98**	**Mean**	**5.72**	
Median	**1.04**	**Median**	**5.89**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	4.40	**GM12**	18.95	
Nasal	16.88	**S54N**	21.60	
Abdo	3.98	**114**	2.94	***P* value** 0.27664
Shin	5.28	**193**	13.17	
2	17.14	**UM9**	10.41	
3	1.65	**176**	2.45	**U value** 30
		177	7.70	
		162	6.93	
Mean	**8.22**	**Mean**	**10.52**	
Median	**4.84**	**Median**	**9.05**	







6L100: 6 hrs-Live-MOI 100:1

6HK100: 6 hrs-Heat killed-MOI 100:1

6CM100: 6 hrs-Conditioned medium-10^7 MRSA in 5 ml growth medium

Figure 4.3: Cytotoxic effects of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 100:1 on KB epithelial cell line six hours post infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.1.4. Cytotoxic effect at multiplicity of infection 100:1 twenty-four hours post-infection

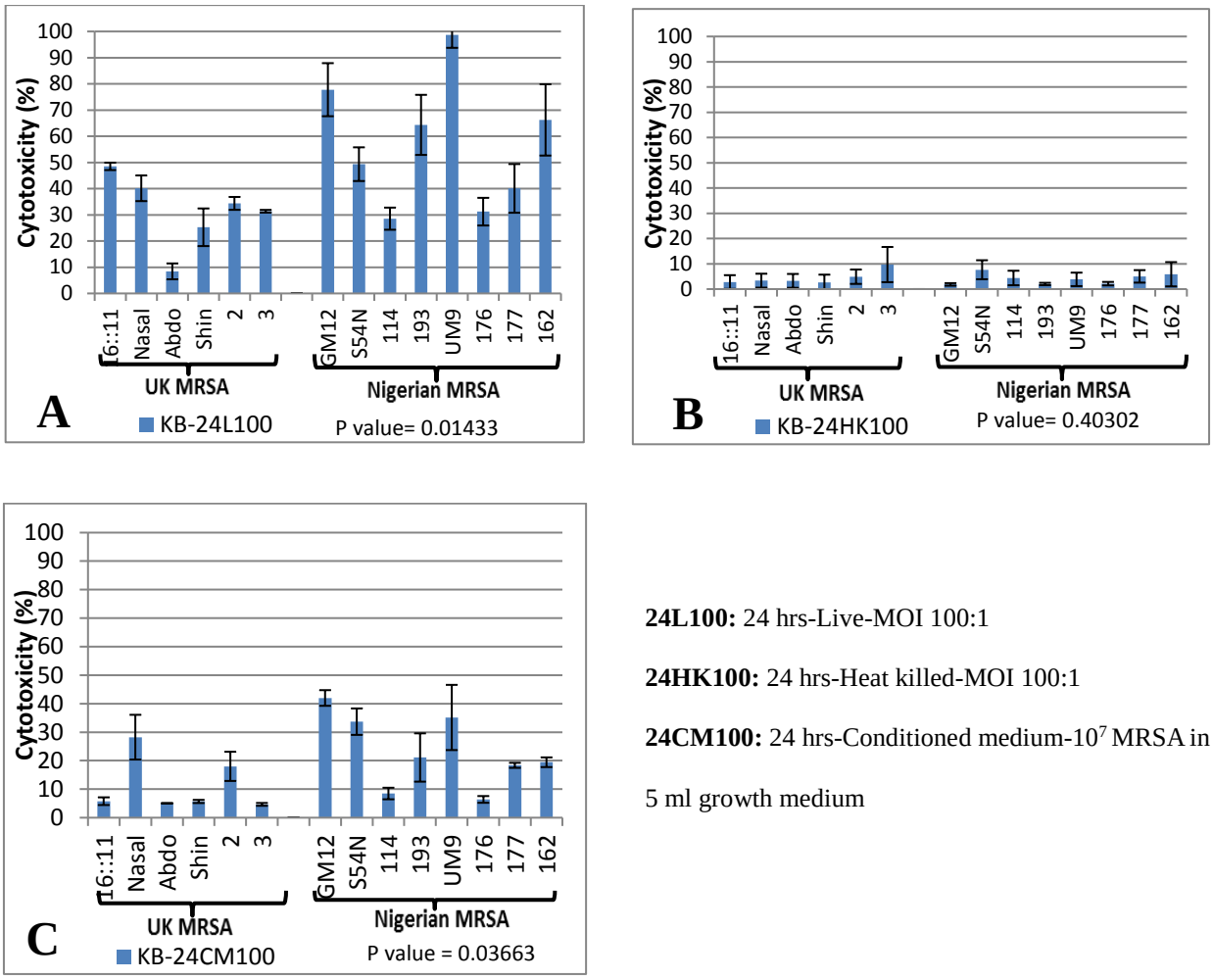
Similar to the results obtained with MOI 100:1 six hrs post-infection, the range of the cytotoxic effect of Nigerian MRSA on KB cells was higher (28.53% - 98.63%) than that of their UK counterparts (8.42% - 48.50%) 24 hrs after infection at MOI 100:1 (Table 4.4). The difference between the mean cytotoxic effect of the live UK and Nigerian MRSA (31.34% and 57.02% respectively) was significant (Figure 4.4A, Table 4.4). Similarly, the median cytotoxic effect of live Nigerian MRSA on KB cells (56.84%) was seen to be significantly (calculated U value [10] < Ucrit [10]) higher than that of UK MRSA (32.87%) (Table 4.4).

When it came to the heat killed MRSA, the cytotoxic effects of the UK MRSA on KB cells was higher (2.64% - 9.70%) than that of the Nigerian MRSA (1.85% - 7.65%) (Table 4.4). Upon comparison, it was seen that the mean toxic effect of the heat killed UK MRSA on KB cells (4.43%) was not significantly higher than that of the Nigerian MRSA (4.10%) (Figure 4.4B, Table 4.4). This was also the case when the median cytotoxic effect of heat killed UK MRSA on KB cells (3.29%) was compared to the median cytotoxic effect of heat killed Nigerian MRSA on KB cells (4.12%) i.e. the difference was insignificant as the calculated U value [23] was > than Ucrit [10] (Table 4.4).

The CM (prepared by inoculating 10^7 CFU bacteria in 5 ml EMEM) of the Nigerian MRSA were seen to exert cytotoxic effects on KB cells at a higher within a higher range (6.40% - 41.99%) compared to the UK MRSA (5.05% - 28.23%) (Table 4.4). As was observed with the toxic effects of live MRSA, the mean toxic effect of the Nigerian CM on KB cells (23.08%) was significantly higher than the mean effect exerted by the CM of UK MRSA (11.24%) (Figure 4.4C, Table 4.4). Similarly, the median cytotoxic effect of the Nigerian CM on KB cells (20.28%) was significantly higher (calculated U value [-1] < Ucrit [10]) than that of UK CM on KB cells (Table 4.4).

Table 4.4: Cytotoxic effects of Live, Heat Killed and Conditioned Media of MRSA at MOI 100:1 on KB epithelial cell 24 hours post-infection. Results are the mean of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on KB epithelial cells at MOI 100:1 24 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	48.50	**GM12**	77.77	
Nasal	40.13	**S54N**	49.34	***P* value** 0.01433
Abdo	8.42	**114**	28.53	
Shin	25.25	**193**	64.34	
2	34.36	**UM9**	98.63	
3	31.37	**176**	31.21	**U value** 10
		177	40.09	
		162	66.23	
Mean	**31.34**	**Mean**	**57.02**	
Median	**32.87**	**Median**	**56.84**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	2.79	**GM12**	1.85	
Nasal	3.33	**S54N**	7.65	***P* value** 0.40302
Abdo	3.25	**114**	4.39	
Shin	2.64	**193**	2.01	
2	4.90	**UM9**	3.84	
3	9.70	**176**	2.15	**U value** 23
		177	5.03	
		162	5.87	
Mean	**4.43**	**Mean**	**4.10**	
Median	**3.29**	**Median**	**4.12**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	5.79	**GM12**	41.99	
Nasal	28.23	**S54N**	33.68	***P* value** 0.03663
Abdo	5.05	**114**	8.47	
Shin	5.69	**193**	21.11	
2	18.01	**UM9**	35.15	
3	4.67	**176**	6.40	**U value** -1
		177	18.36	
		162	19.45	
Mean	**11.24**	**Mean**	**23.08**	
Median	**5.74**	**Median**	**20.28**	



24L100: 24 hrs-Live-MOI 100:1

24HK100: 24 hrs-Heat killed-MOI 100:1

24CM100: 24 hrs-Conditioned medium-10^7 MRSA in 5 ml growth medium

Figure 4.4: Cytotoxic effects of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 100:1 on KB epithelial cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.2. Cytotoxic effects of MRSA on A549 lung cell line

4.3.1.2.1. Cytotoxic effects at multiplicity of infection 10:1 six hours post-infection

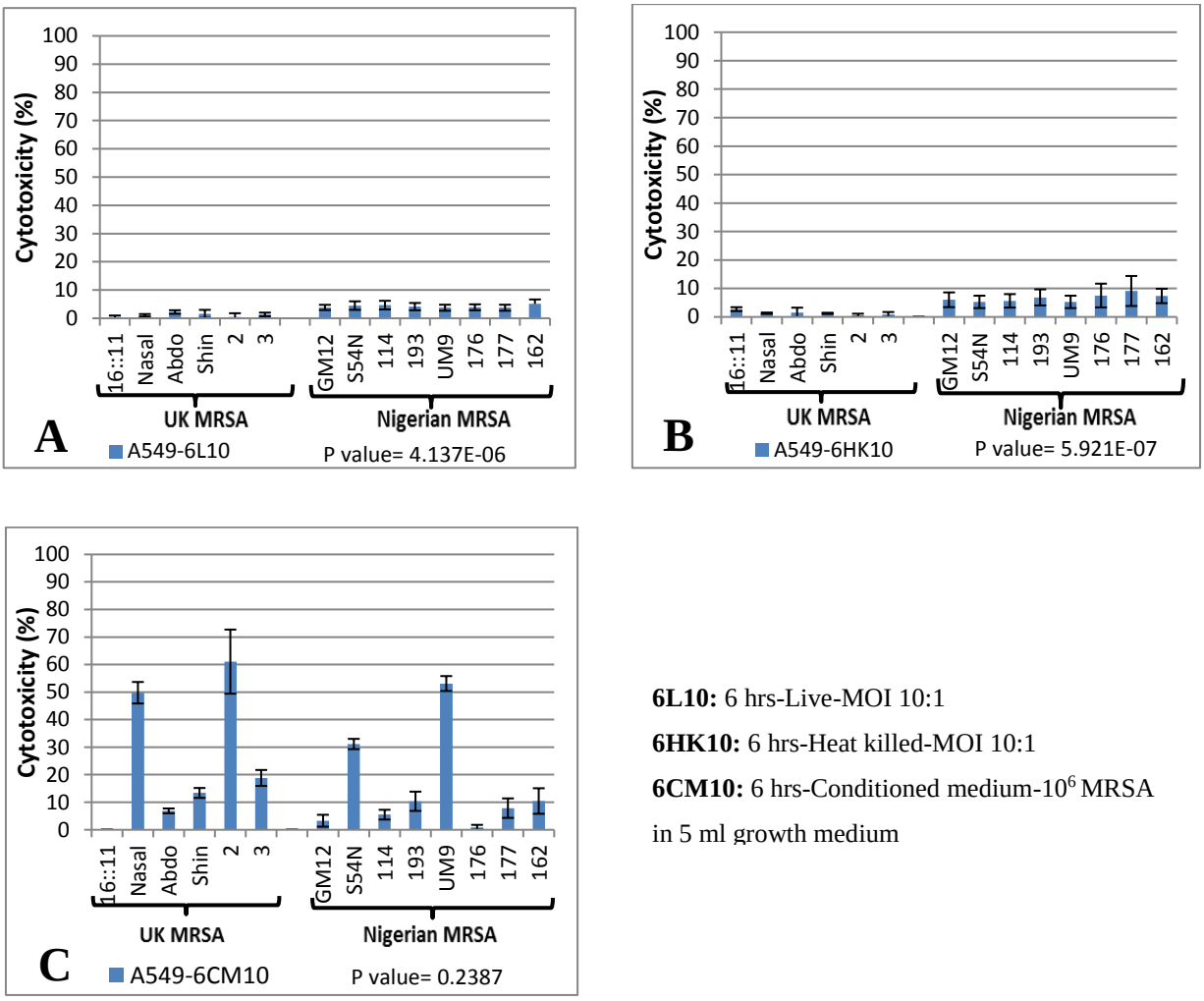
Six hours following infection of A549 cells at the rate of 10 bacteria to 1 cell, live Nigerian MRSA were seen to have exerted higher toxic effects (range of 3.73% - 5.08%) than the live UK MRSA (range of 0.40% - 2.21%) (Table 4.5). On comparison, the results showed that the mean cytotoxic effect of the Nigerian MRSA (4.19%) was significantly higher than that of the UK MRSA (1.17%) (Figure 4.5A, Table 4.5). A significant difference was also observed between the median cytotoxic effects of live UK and Nigerian MRSA on A549 cells (1.18% and 4.00%) respectively (calculated U value [1] < Ucrit [10]) (Table 4.5).

Similarly, heat killed Nigerian MRSA at MOI 10:1 and 6 hrs post-infection were seen to exert cytotoxic effects on A549 cells within a higher range (5.28% - 9.12%) than their UK counterparts (0.55% - 2.73%) (Table 4.5). Differences in the mean and median cytotoxic effects of the heat killed UK and Nigerian MRSA (Means: UK - 1.37% and Nigerian - 6.64%; Medians: UK - 1.30% and Nigerian - 6.44%) were also seen to be significant i.e. *P* value = 5.921E-07; calculated U value [1] < Ucrit [10] (Figure 4.5B, Table 4.5).

On the other hand, results from this study showed that the CM of the UK MRSA isolates exerted cytotoxic effects on A549 cells within a range of 0.00% - 50.60%, while that of the Nigerian MRSA was within 0.90% - 53.11% (Table 4.5). In this instance, the mean as well as median cytotoxic effects exerted by the CM of the UK MRSA on the cells (22.93% and 16.11% respectively) were not significantly higher than those exerted by the CM of the Nigerian isolates (15.32% and 9.11% respectively) i.e. *P* value = 0.2387; calculated U value [18] > Ucrit [10] (Table 4.5, Figure 4.5C).

Table 4.5: Cytotoxic effects of Live, Heat Killed and Conditioned Media of MRSA at MOI 10:1 on A549 lung cell line 6 hours post-infection. Results are the mean of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on A549 cells at MOI 10:1 6 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	0.40	**GM12**	3.83	***P* value** 4.137E-06
Nasal	1.03	**S54N**	4.49	
Abdo	2.21	**114**	4.66	
Shin	1.54	**193**	4.12	
2	0.49	**UM9**	3.73	
3	1.33	**176**	3.88	**U value** 1
		177	3.73	
		162	5.08	
Mean	**1.17**	**Mean**	**4.19**	
Median	**1.18**	**Median**	**4.00**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	2.73	**GM12**	6.02	***P* value** 5.921E-07
Nasal	1.32	**S54N**	5.28	
Abdo	1.52	**114**	5.65	
Shin	1.28	**193**	6.86	
2	0.55	**UM9**	5.28	
3	0.83	**176**	7.52	**U value** 1
		177	9.12	
		162	7.37	
Mean	**1.37**	**Mean**	**6.64**	
Median	**1.30**	**Median**	**6.44**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	0.00	**GM12**	3.29	***P* value** 0.2387
Nasal	50.60	**S54N**	31.15	
Abdo	10.70	**114**	5.50	
Shin	13.38	**193**	10.35	
2	44.07	**UM9**	53.11	
3	18.83	**176**	0.90	**U value** 18
		177	7.86	
		162	10.45	
Mean	**22.93**	**Mean**	**15.32**	
Median	**16.11**	**Median**	**9.11**	



6L10: 6 hrs-Live-MOI 10:1

6HK10: 6 hrs-Heat killed-MOI 10:1

6CM10: 6 hrs-Conditioned medium-10^6 MRSA in 5 ml growth medium

Figure 4.5: Cytotoxic effect of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 10:1 on A549 lung cell line 6 hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.2.2. Cytotoxic effects at multiplicity of infection 10:1 twenty-four hours post-infection

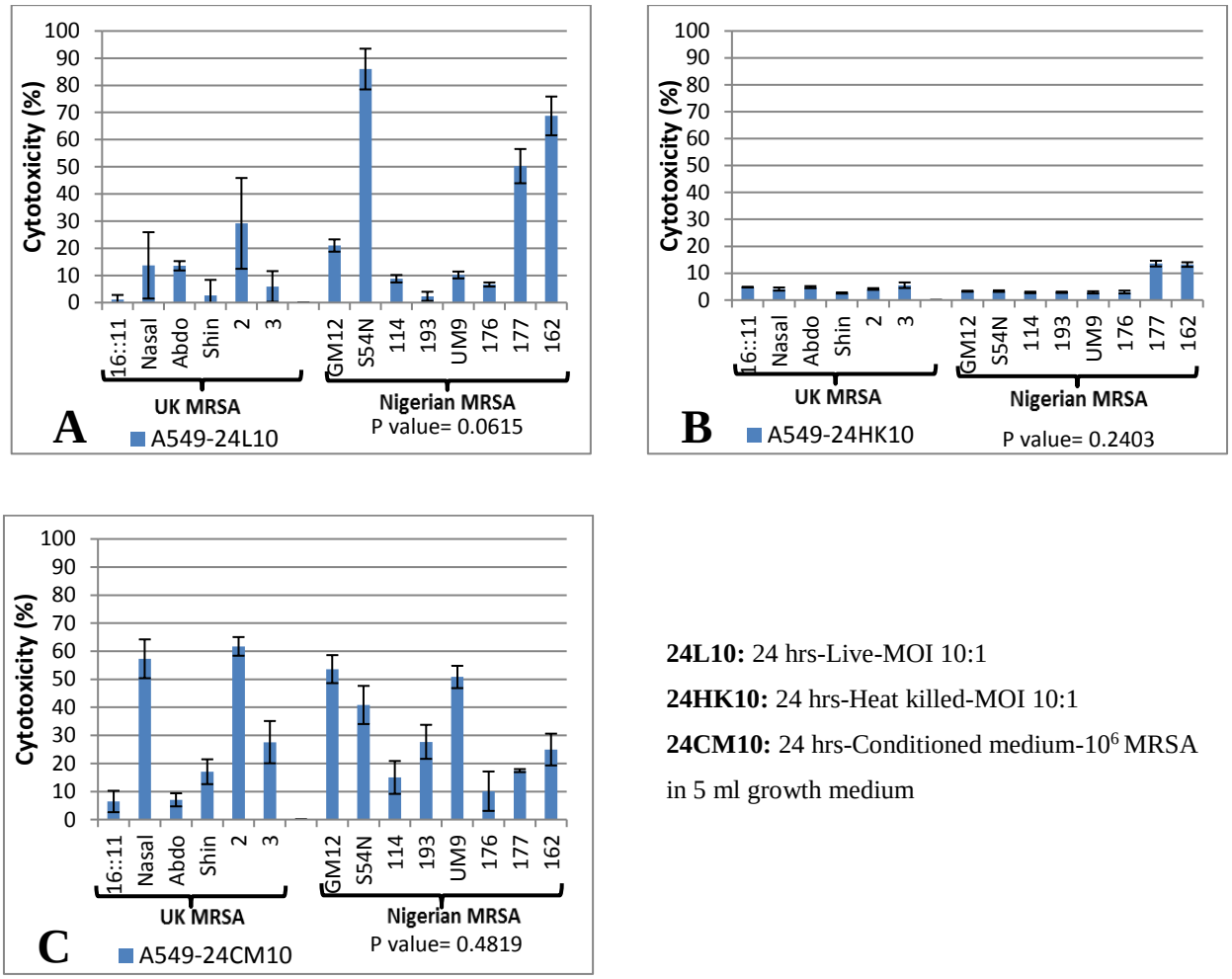
Twenty-four hours following infection at MOI 10:1, live Nigerian MRSA exerted cytotoxic effects on A549 cells within a higher range (2.29% - 86.00%) compared with the UK MRSA (1.15% - 29.16%) (Table 4.6). However, although the mean toxic effect of the live Nigerian MRSA (31.73%) was higher than that of the UK MRSA (11.02%), the observed difference was not significant (Figure 4.6A, Table 4.6). The median cytotoxic effect of live Nigerian MRSA on A549 cells (15.59%) was also insignificantly higher than that of live UK MRSA (9.73%) i.e. calculated U value [15] > Ucrit [10] (Table 4.6).

The cytotoxic effects of UK MRSA on A549 cells 24 hrs following infection at MOI 10:1 ranged from 2.63% - 5.52% while those of Nigerian MRSA ranged from 2.86% - 13.56% (Table 4.6). Although the mean cytotoxic effect of the Nigerian MRSA (5.62%) was higher than that of the UK MRSA (4.33%), the difference was not significant (Table 4.6, Figure 4.6B). On the other hand, the median cytotoxic effect of heat killed UK MRSA (4.45%) was seen to be higher than that of heat killed Nigerian MRSA (3.15%); this difference was however insignificant as the calculated U value [18] was > Ucrit [10] (Table 4.6).

As was the case with heat killed MRSA isolates, the mean cytotoxic effect exerted by the CM of Nigerian MRSA (30.06%) was not significantly higher than that of the UK MRSA (29.52%) (Figure 4.6C, Table 4.6). In this instance, although the median cytotoxic effect of the Nigerian CM on A549 cells was higher (26.33%) than that of the UK CM on A549 cells (22.31%), the difference was insignificant (calculated U value [22] > Ucrit [10]) (Table 4.6).

Table 4.6: Cytotoxic effects of Live, Heat Killed and Conditioned Media of MRSA at MOI 10:1 on A549 lung cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on A549 cells at MOI 10:1 24 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	1.15	**GM12**	21.01	
Nasal	13.70	**S54N**	86.00	***P* value** 0.0615
Abdo	13.54	**114**	8.78	
Shin	2.67	**193**	2.29	
2	29.16	**UM9**	10.16	
3	5.92	**176**	6.66	**U value** 15
		177	50.24	
		162	68.73	
Mean	**11.02**	**Mean**	**31.73**	
Median	**9.73**	**Median**	**15.59**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	4.81	**GM12**	3.29	
Nasal	4.10	**S54N**	3.33	***P* value** 0.2403
Abdo	4.81	**114**	2.84	
Shin	2.63	**193**	2.89	
2	4.12	**UM9**	2.86	
3	5.52	**176**	3.00	**U value** 18
		177	13.56	
		162	13.20	
Mean	**4.33**	**Mean**	**5.62**	
Median	**4.45**	**Median**	**3.15**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	6.44	**GM12**	53.61	
Nasal	57.29	**S54N**	40.84	***P* value** 0.4819
Abdo	7.02	**114**	15.01	
Shin	17.04	**193**	27.70	
2	61.72	**UM9**	50.82	
3	27.58	**176**	10.10	**U value** 22
		177	17.44	
		162	24.95	
Mean	**29.52**	**Mean**	**30.06**	
Median	**22.31**	**Median**	**26.33**	



24L10: 24 hrs-Live-MOI 10:1

24HK10: 24 hrs-Heat killed-MOI 10:1

24CM10: 24 hrs-Conditioned medium-10^6 MRSA in 5 ml growth medium

Figure 4.6: Cytotoxic effect of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 10:1 on A549 lung cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.2.3. Cytotoxic effects at multiplicity of infection 100:1 six hours post-infection

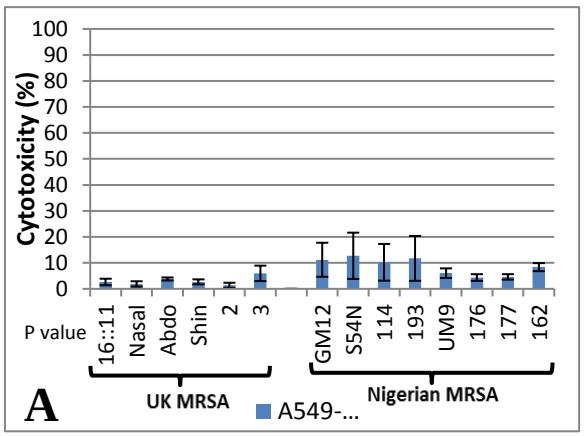
Six hours after infection at MOI 100:1, the cytotoxic effects of live UK MRSA on A549 cells ranged from 1.38% - 5.96% while those of live Nigerian MRSA ranged from 4.36% - 12.71% (Table 4.7). The mean cytotoxic effect of the live Nigerian MRSA (8.65%) was seen to be significantly higher than that of the live UK MRSA isolates (3.06%) (Figure 4.7A, Table 4.7). A comparison of medians also showed that the median cytotoxic effect of live Nigerian MRSA on A549 cells (9.29%) was also significantly higher than that of UK MRSA (2.67%) i.e. calculated U value [2] < Ucrit [10] (Table 4.7).

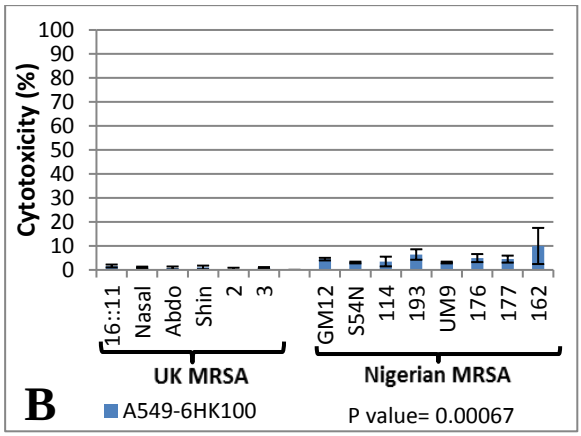
Heat killed Nigerian MRSA exerted cytotoxic effects within a higher range (3.08% - 9.98%) than their UK counterparts (range from 0.78% - 1.63%) (Table 4.7). As was observed with the live MRSA isolates, the mean and median toxic effects of heat killed Nigerian MRSA (5.00% and 4.50% respectively) were significantly higher than those of the UK isolates (0.92% and 0.89% respectively) i.e. *P* value = 0.00067; calculated U value [1] < Ucrit [10] (Figure 4.7B, Table 4.7).

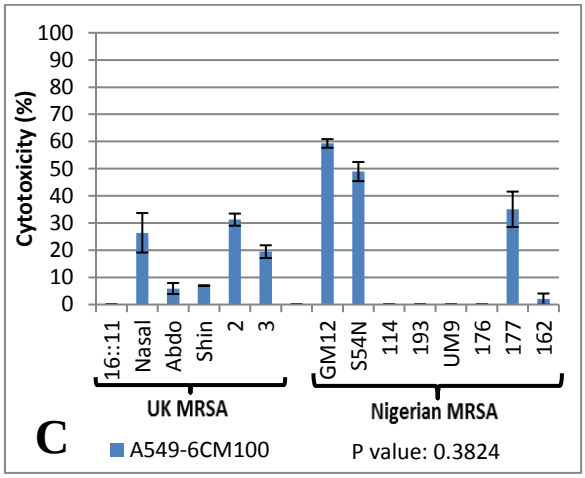
Although the mean toxic effect of the CM of the Nigerian isolates (18.16%) was higher than that of the UK isolates (14.99%), the difference was insignificant (*P* value = 0.3824) (Figure 4.7C, Table 4.7). Furthermore, a comparison of medians showed that the median cytotoxic effect of the UK CM on A549 cells (13.21%) was insignificantly higher than that of the Nigerian CM on A549 cells (1.02%) i.e. calculated U value [23] > Ucrit [10] (Table 4.7).

Table 4.7: Cytotoxic effects of Live, Heat Killed and Conditioned Media of MRSA at MOI 100:1 on A549 lung cell line 6 hours post-infection. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on A549 cells at MOI 100:1 6 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	2.65	**GM12**	11.19	
Nasal	1.89	**S54N**	12.71	***P* value** 0.0009
Abdo	3.80	**114**	10.22	
Shin	2.69	**193**	11.72	
2	1.38	**UM9**	6.04	
3	5.96	**176**	4.36	**U value** 2
		177	4.60	
		162	8.36	
Mean	**3.06**	**Mean**	**8.65**	
Median	**2.67**	**Median**	**9.29**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	1.63	**GM12**	4.49	
Nasal	1.00	**S54N**	3.08	***P* value** 0.00067
Abdo	0.79	**114**	3.49	
Shin	1.02	**193**	6.42	
2	0.28	**UM9**	3.08	
3	0.78	**176**	4.93	**U value** 1
		177	4.50	
		162	9.98	
Mean	**0.92**	**Mean**	**5.00**	
Median	**0.89**	**Median**	**4.50**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	0.00	**GM12**	59.28	
Nasal	26.40	**S54N**	48.92	***P* value** 0.3824
Abdo	5.90	**114**	0.00	
Shin	6.96	**193**	0.00	
2	31.24	**UM9**	0.00	
3	19.46	**176**	0.00	**U value** 23
		177	35.04	
		162	2.03	
Mean	**14.99**	**Mean**	**18.16**	
Median	**13.21**	**Median**	**1.02**	







6L100: 6 hrs-Live-MOI 100:1

6HK100: 6 hrs-Heat killed-MOI 100:1

6CM100: 6 hrs-Conditioned medium-10^7 MRSA in 5 ml growth medium

Figure 4.7: Cytotoxic effect of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 100:1 on A549 lung cell line 6 hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.1.2.4. Cytotoxic effects at multiplicity of infection 100:1 twenty-four hours post infection

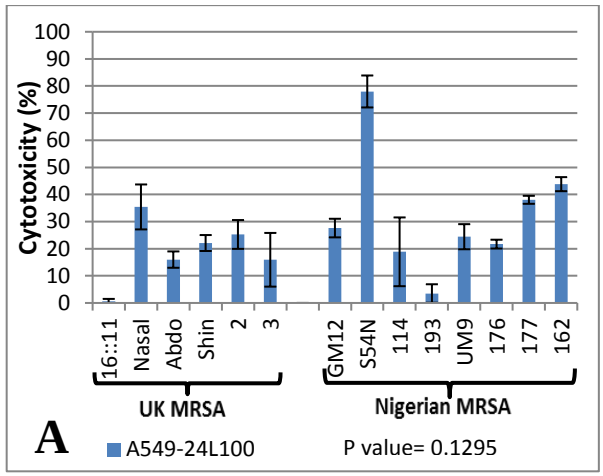
Among all the isolates (UK and Nigerian), it was Nigerian isolate S54N that exerted the highest cytotoxic effect on A549 cells 24 hrs following infection at MOI 100:1 (Table 4.8). It was also a Nigerian isolate (193) that exerted the least toxic effect of all the isolates under the same conditions (Table 4.8). Nevertheless, the mean and median toxic effects of the Nigerian isolates (32.00% and 26.03% respectively) were higher (insignificantly) than those of the UK isolates (21.11% and 19.05% respectively) i.e. *P* value = 0.1295; calculated U value [14] > Ucrit [10] (Figure 4.8A, Table 4.8).

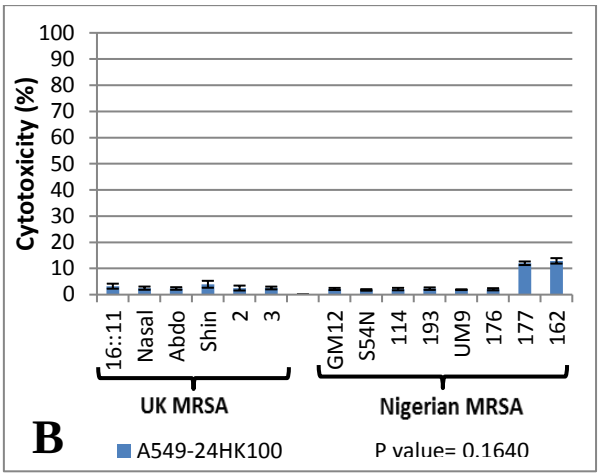
Similar to what was seen with the live MRSA, it was heat killed Nigerian isolates that exerted the highest and least toxic effects (isolates 162 and S54N respectively) on A549 cells 24 hrs after infection with MOI 100:1 (Table 4.8). In the same vein, the mean and median toxic effects of heat killed Nigerian MRSA (4.65%) was not significantly higher (*P* value = 0.1640) than that of the UK isolates (2.84%) (Figure 4.8B, Table 4.8). Although the median cytotoxic effect of heat killed UK MRSA on A549 cells (2.53%) was higher than that of heat killed Nigerian MRSA, the difference was not significant as the calculated U value [12] was > than Ucrit [10] (Table 4.8).

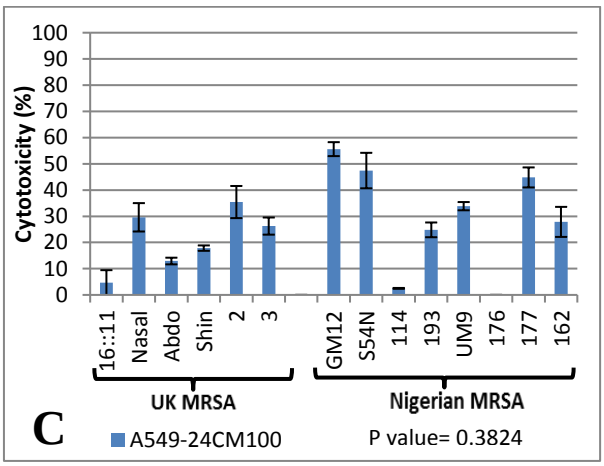
It was also the CM of Nigerian isolates that were the most and least toxic, of all the isolates (UK and Nigerian) on A549 cells (isolates GM12 and 176 respectively) (Table 4.8). The mean and median cytotoxic effects of the CM of the Nigerian isolates (29.60% and 30.85% respectively) were not significantly higher than those of the UK MRSA (16.20% and 22.05% respectively) i.e. *P* value = 0.0572; calculated U value [18] > Ucrit [10] (Figure 4.8C, Table 4.8).

Table 4.8: Cytotoxic effects of Live, Heat Killed and Conditioned Media of MRSA at MOI 100:1 on A549 lung cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate. The highest and lowest percentages per series are highlighted yellow and turquoise respectively. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test. Difference between the medians of two data sets were considered significant if the computed U value was $\leq$ Ucrit (Mann-Whitney U test).

Cytotoxic effects of MRSA on A549 cells at MOI 100:1 24 hrs post-infection				
Live bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	4.62	**GM12**	27.62	
Nasal	29.60	**S54N**	77.99	***P* value** 0.1295
Abdo	12.92	**114**	18.87	
Shin	17.84	**193**	3.50	
2	35.42	**UM9**	24.43	
3	26.26	**176**	21.70	**U value** 14
		177	38.04	
		162	43.81	
Mean	**21.11**	**Mean**	**32.00**	
Median	**19.05**	**Median**	**26.03**	
Heat killed bacteria				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	3.23	**GM12**	2.17	
Nasal	2.47	**S54N**	1.80	***P* value** 0.1640
Abdo	2.36	**114**	2.12	
Shin	3.95	**193**	2.29	
2	2.52	**UM9**	1.89	
3	2.54	**176**	2.05	**U value 12**
		177	11.99	
		162	12.90	
Mean	**2.84**	**Mean**	**4.65**	
Median	**2.53**	**Median**	**2.15**	
Bacterial conditioned media				
UK		**Nigerian**		
Isolates	**Cytotoxic effects (%)**	**Isolates**	**Cytotoxic effects (%)**	
16::11	4.62	**GM12**	55.59	
Nasal	18.28	**S54N**	47.42	***P* value** 0.0572
Abdo	12.92	**114**	2.50	
Shin	17.84	**193**	24.81	
2	22.63	**UM9**	33.84	
3	20.88	**176**	0.00	**U value** 18
		177	44.81	
		162	27.85	
Mean	**16.20**	**Mean**	**29.60**	
Median	**22.05**	**Median**	**30.85**	







24L100: 24 hrs-Live-MOI 100:1

24HK100: 24 hrs-Heat killed-MOI 100:1

24CM100: 24 hrs-Conditioned medium-10^7 MRSA in 5 ml growth medium

Figure 4.8: Cytotoxic effect of Live **(A)**, Heat Killed **(B)** and Conditioned Media **(C)** of MRSA at MOI 100:1 on A549 lung cell line 24 hours post-infection. Results are the average of three independent experiments performed in duplicate; error bars represent standard error. Values of $P < 0.05$ were accepted as significantly different based on the 1-tailed unpaired t-test.

4.3.2. Distribution of apoptotic and necrotic cells following MRSA infection

Flow cytometry was used to determine the distribution of apoptotic and necrotic cells following infection of KB epithelial and A549 lung cell lines with live UK MRSA (MOIs 10:1 and 100:1) as described in section 4.2.6.

Flow cytometry is a system for sensing cells as they move in a liquid stream through a laser/light beam past a sensing area (Macey 2007). It measures the optical and fluorescence characteristics of single cells (Brown and Wittwer 2000). Furthermore, cells are analysed and differentiated based on granularity, size and whether the cell is fluorescently labelled with either antibodies or dyes (Macey 2007). The FITC Annexin V (Fluorochrome-labelled) dye used in this study binds the phospholipid phosphatidylserine (PS). PS is normally only found on the intracellular leaflet of the plasma membrane in healthy cells. However, during early apoptosis, membrane integrity is lost and PS translocates to the external leaflet. Thus, FITC Annexin V aids identification of apoptotic cells. The FITC Annexin V was used in conjunction with Zombie NIR (a fluorescent dye non-permeant to live cells) to aid differentiation between apoptotic and necrotic cells.

Four populations of cells were distinguished by the flow cytometer i.e. those that were not stained by either FITC Annexin V (FITC) or Zombie NIR (ZB) - viable cells, those that were stained by FITC but not Zombie – early apoptotic cells, those that were stained by both FITC and Zombie – late apoptotic cells and finally, the population of cells that were not stained by FITC but were stained by Zombie – necrotic cells. Results (dot plots) generated by the flow cytometer are as exemplified in the Figure 4.9 A, with each quadrant representing a given population of the stained cells. Percentages of each population were then obtained and used for statistical analysis. The forward versus side scatter (SSC vs FSC) gating strategy was applied in order to exclude debris, air bubbles and laser noise.

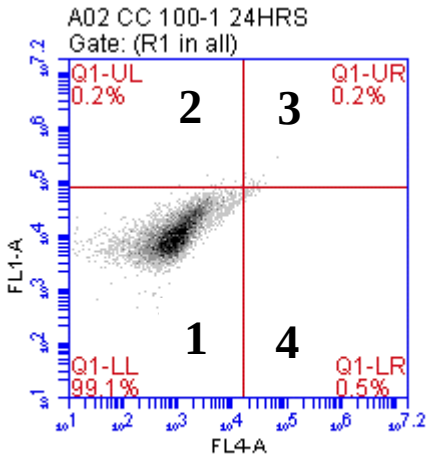


Figure 4.9A: Dot plot exemplifying results generated by the flow cytometer. **Quadrant 1:** Viable cells (FITC -ve, ZB -ve); **Quadrant 2:** Early apoptotic cells (FITC +ve ZB -ve); **Quadrant 3:** Late apoptotic cells (FITC +ve, ZB +ve); **Quadrant 4:** Necrotic cells (FITC -ve, ZB **+ve).** Picture shows distribution of uninfected KB cells after staining.

FITC: FITC Annexin V

ZB: Zombie NIR

+ve: Positive

-ve: Negative

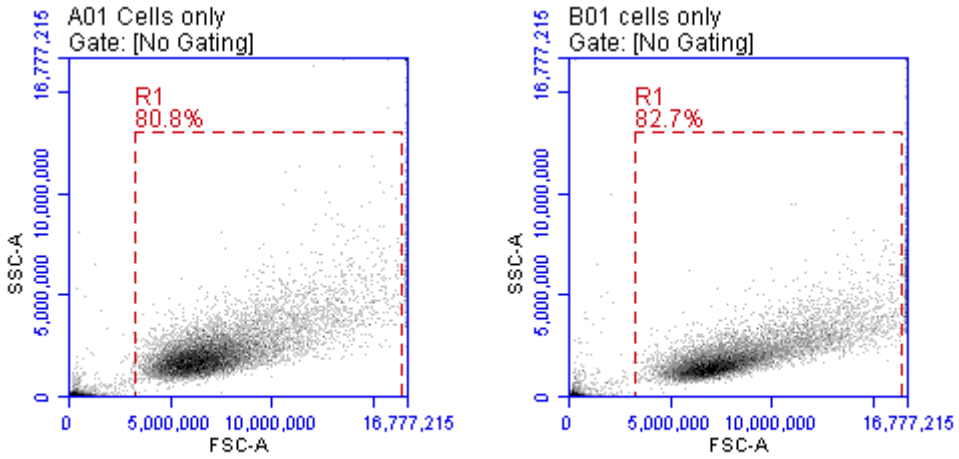


Figure 4.9B: Representative side vs forward scatter density plots of MRSA infected mammalian cells generated in the course of this study. Each dot or point on the plots represent individual particles that have passed through the laser. A gate has been applied to remove debris.

4.3.2.1. Distribution of infected KB epithelial cells

4.3.2.1.1. Distribution of cells six hours post-infection at MOI 10:1

Six hrs post-infection at MOI 10:1, results showed that majority (≥ 55%) of KB cells infected with each live MRSA isolate (six UK and eight Nigerian) were in the early phase of apoptosis (Figure 4.10, Table 4.9). Under the same conditions, the results obtained also show that > 25% but < 50% of cells infected with the Nigerian isolates UM9, 176 and 177 were in the late apoptotic stage of death, an indication that these isolates were more toxic. These results are in agreement with the results obtained from the cytotoxicity assays where it was seen that Nigerian isolates UM9, 176 and 177 were the most toxic on KB cells at MOI 10:1 at 6 hrs post-infection (4.3.1.1.1). The results further show that six hrs post-infection, < 1.5% of KB cells infected with each MRSA isolate (UK and Nigerian) were necrotic. These results are further summarised in Table 4.9.

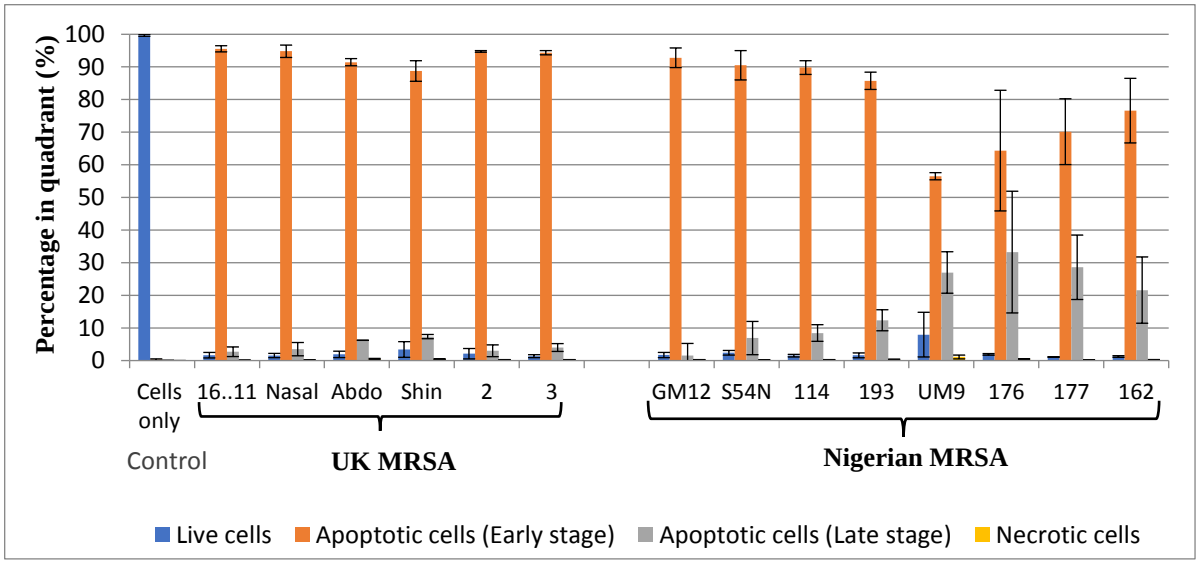


Figure 4.10: Distribution of apoptotic and necrotic KB cells 6 hours following infection with live MRSA at MOI 10:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

4.3.2.1.2. Distribution of cells twenty-four hours post-infection at MOI 10:1

Results from the cytotoxicity assays show that at MOI 10:1, the MRSA isolates exerted greater cytotoxic effects on the KB cells after 24 hrs (4.3.1.1.2) compared with their cytotoxic effect after 6 hrs (4.3.1.1.1). Similarly, flow cytometry results 24 hrs post-infection at MOI 10:1 show that there is an increase in the proportion of infected KB cells that had transited from the early apoptotic to the late apoptotic phase (Table 4.9). This is because it was only when seven isolates (UK isolates 16/11, Abdo, Shin and Nigerian isolates S54N, 114, 176, 177) infected KB cells that > 50% of the infected cells were in the early stages of apoptosis 24 hrs post-infection (Figure 4.11). That was not the case 6 hrs post-infection when it was seen that > 55% of the infected cells were in the early apoptotic phase following infection with all the isolates (Figure 4.10).

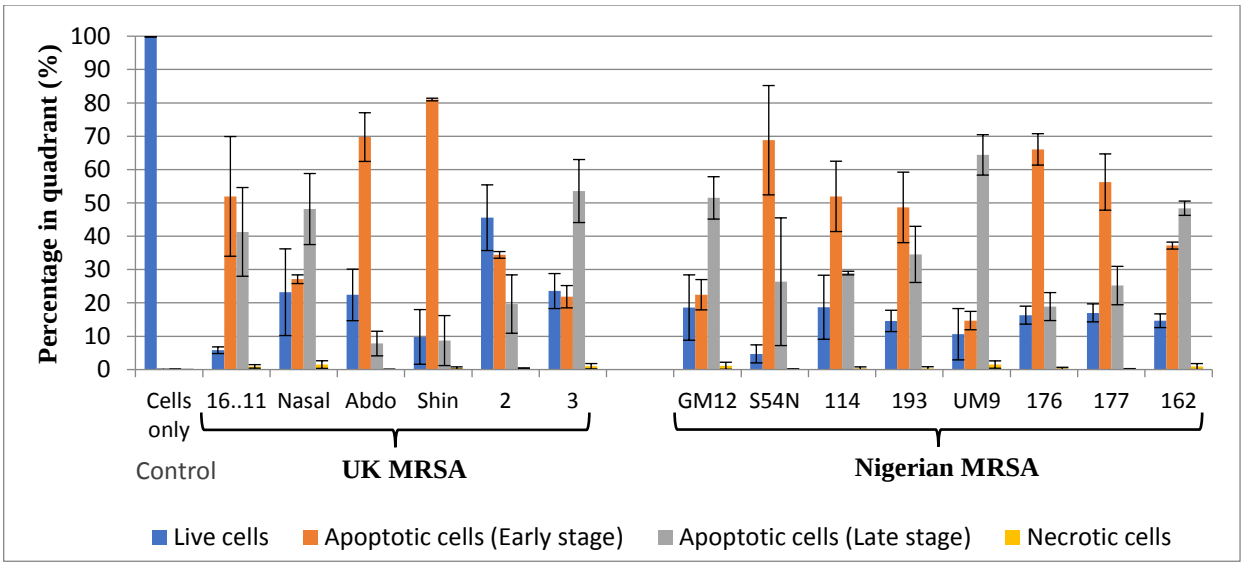


Figure 4.11: Distribution of apoptotic and necrotic KB cells 24 hours following infection with live MRSA at MOI 10:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

The results also showed an increase in the proportions of live cells 24 hrs post-infection with all the isolates (Figure 4.11, Table 4.11). The proportions of necrotic cells 24 hrs post-infection with all of the isolates were ≤ 1.5% (Figure 4.11, Table 4.9).

Table 4.9: Proportional distribution of viable, apoptotic and necrotic cells 6 and 24 hours following infection of KB epithelial cells with MRSA at MOI 10:1. Results are the mean of three independent experiments performed in duplicate.

MRSA Isolate (MOI 10:1)	Proportion of Viable cells (%)		Proportion of apoptotic cells % (Early stage)		Proportion of apoptotic cells % (Late stage)		Proportion of necrotic cells %	
	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs
UK								
16..11	1.65	5.80	95.55	51.95	2.70	41.30	0.00	0.95
Nasal	1.55	23.20	94.80	27.10	3.50	48.15	0.10	1.50
Abdo	1.90	22.40	91.45	69.73	6.20	7.80	0.15	0.07
Shin	3.40	9.80	88.75	81.00	7.35	8.70	0.50	0.45
2	2.10	45.55	94.75	34.40	3.00	19.65	0.45	0.35
3	1.35	23.55	94.35	21.85	4.00	53.55	0.25	1.00
Nigerian								
GM12	1.70	18.60	92.80	22.45	1.50	51.50	0.25	1.15
S54N	2.40	4.70	90.50	68.80	6.90	26.35	0.15	0.10
114	1.50	18.70	89.80	51.95	8.45	28.95	0.25	0.40
193	1.60	14.60	85.75	48.65	12.35	34.55	0.30	0.40
UM9	7.95	10.60	56.50	14.70	27.00	64.40	1.10	1.50
176	1.90	16.33	64.35	66.05	33.25	18.90	0.45	0.37
177	1.10	17.00	70.15	56.25	28.60	25.20	0.15	0.17
162	1.25	14.65	76.60	37.20	21.60	48.40	0.30	0.90

4.3.2.1.3. Distribution of cells six hours post-infection at MOI 100:1

Similar to results obtained in section 4.3.2.1.1, results showed that after 6 hrs, > 50% of KB cells infected with each MRSA isolate at MOI 100:1 were in the early apoptotic phase of death (Figure 4.12, Table 4.10). It was only when three isolates (Nigerian isolates UM9, 176 and 177) infected KB cells, under the present conditions, that the results show >15% but <50% of the cells being in the late apoptotic death phase. At this time, only < 1% of cells infected with each MRSA isolate were seen to be necrotic.

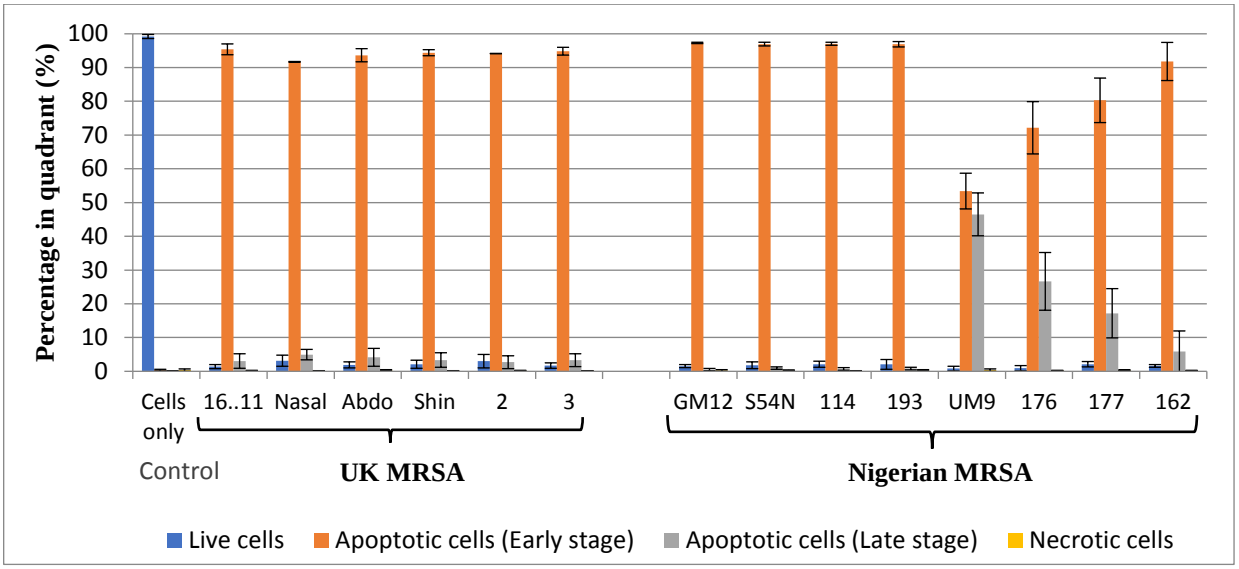


Figure 4.12: Distribution of apoptotic and necrotic KB cells 6 hours following infection with live MRSA at MOI 100:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

4.3.2.1.4. Distribution of cells twenty-four hours post-infection at MOI 100:1

As in section 4.3.2.1.2, the results also show an increase in the proportion of infected cells that had transitioned from the early apoptotic to the late apoptotic phase of death 24 hrs after infection with MRSA at MOI 100:1 (Figure 4.13, Table 4.10). This is also in agreement with the cytotoxicity results that show that at MOI 100:1, the MRSA isolates (UK and Nigerian) exert a higher cytotoxic effect on KB cells after 24 hrs than after 6 hrs (Section 4.3.1.1.4, Table 4.4, Figure 4.4**)**

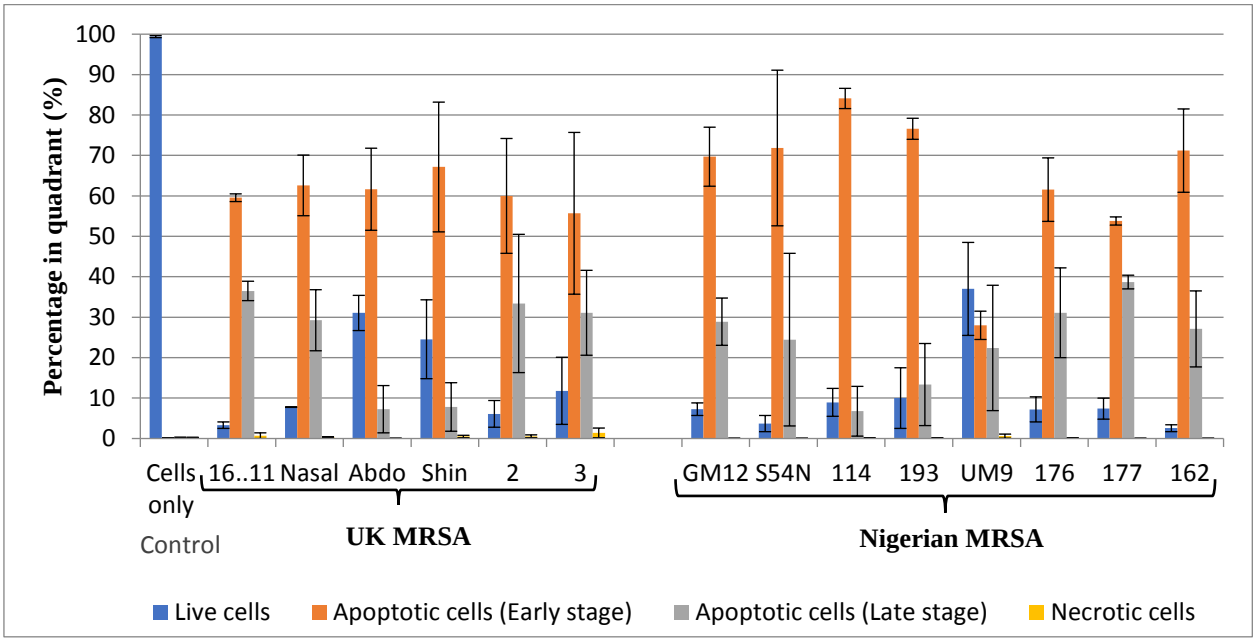


Figure 4.13: Distribution of apoptotic and necrotic KB cells 24 hours following infection with live MRSA at MOI 100:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

The results also showed an increase in the proportions of live cells 24 hrs post-infection with all the isolates. The proportions of necrotic cells 24 hrs post-infection were seen to be < 1% (Figure 4.13, Table 4.10).

Table 4.10: Proportional distribution of viable, apoptotic and necrotic cells 6 and 24 hours following infection of KB epithelial cells with MRSA at MOI 100:1. Results are the mean of three independent experiments performed in duplicate.

MRSA Isolate (MOI 100:1)	Proportion of Viable cells (%)		Proportion of apoptotic cells % (Early stage)		Proportion of apoptotic cells % (Late stage)		Proportion of necrotic cells %	
	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs
UK								
16..11	1.40	3.30	95.40	59.55	3.05	36.50	0.20	0.70
Nasal	3.15	7.75	91.70	62.60	4.95	29.25	0.15	0.40
Abdo	1.90	31.05	93.65	61.65	4.15	7.25	0.30	0.05
Shin	2.10	24.55	94.40	67.15	3.35	7.80	0.20	0.50
2	3.00	6.10	94.15	60.00	2.70	33.40	0.20	0.55
3	1.70	11.80	94.85	55.70	3.30	31.10	0.15	1.40
Nigerian								
GM12	1.55	7.25	97.30	69.70	0.50	28.90	0.25	0.05
S54N	1.80	3.70	96.95	71.85	0.95	24.45	0.25	0.05
114	2.10	8.95	97.05	84.10	0.70	6.75	0.10	0.10
193	2.05	10.00	96.90	76.60	0.80	13.35	0.30	0.10
UM9	0.80	37.00	53.40	28.00	46.50	22.40	0.35	0.60
176	0.90	7.20	72.15	61.55	26.65	31.10	0.25	0.10
177	2.10	7.40	80.30	53.80	17.20	38.70	0.35	0.05
162	1.60	2.55	91.80	71.20	5.90	27.10	0.25	0.05

4.3.2.2. Distribution of infected A549 lung cells

4.3.2.2.1. Distribution of cells six hours post-infection at MOI 10:1

Results obtained show that the 6 hrs post-infection at MOI 10:1, majority of A549 cells (>70%) infected with each UK isolate were in the early apoptotic death phase and that in each instance, only < 10% of the infected cells were in the late stage of apoptosis (Figure 4.14, Table 4.11). However, it can be seen from the results that > 10% but < 21% of A549 cells infected by each Nigerian MRSA isolate (except isolates GM12 and S54N) were in the late apoptotic phase of death, an indication that after 6 hrs, the Nigerian isolates could be more toxic than the UK isolates on A549 cells at MOI 10:1. Results from the cytotoxicity assays show Nigerian MRSA to be more toxic on A549 cells at MOI 10:1 after 6 hrs (4.3.1.2.1), thereby confirming speculations from this study (flow cytometry). The proportions of necrotic cells at this time were seen to range between 0.65% - 4.70%.

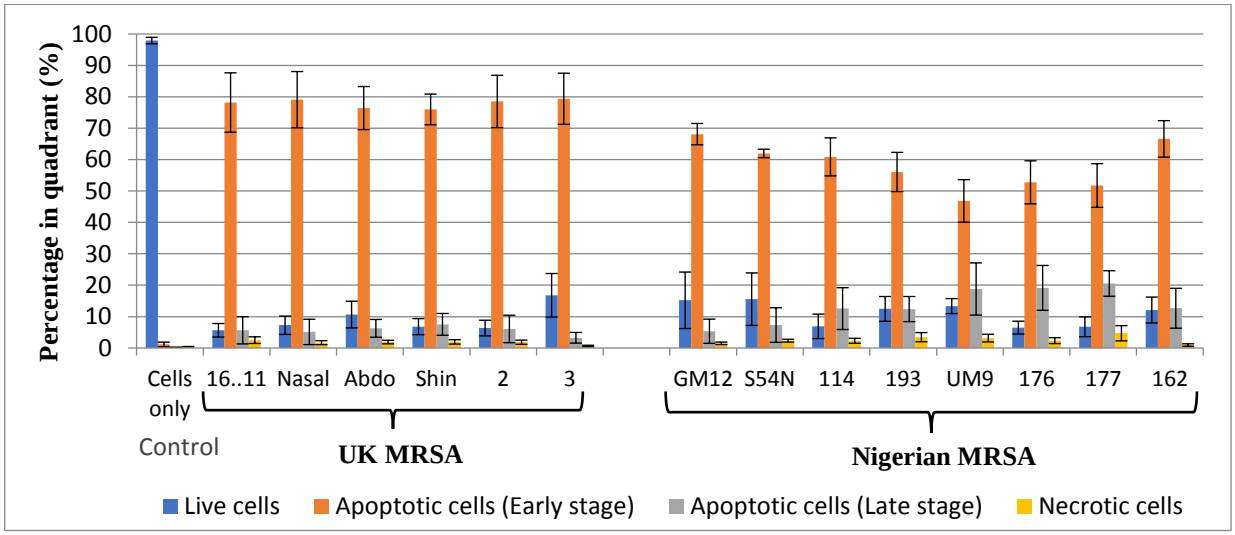


Figure 4.14: Distribution of apoptotic and necrotic A549 lung cells 6 hours following infection with live MRSA at MOI 10:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

4.3.2.2.2. Distribution of cells twenty-four hours post-infection at MOI 10:1

Twenty-four hrs following infection at MOI 10:1, > 10% but < 55% of the A549 cells infected with each Nigerian isolate and each of five UK isolates (except UK isolate 2) were seen to be viable (Figure 4.15, Table 4.11), which is an overall increase from what was seen 6 hrs post-infection. This is because the proportions of viable cells 6 hrs post-infection at MOI 10:1 with all the isolates ranged from 5.65% - 16.80% (Figure 4.14, Table 4.11). Results show that >10% but < 60% of A549 cells infected by all but one Nigerian isolate (114) were in the late apoptotic stage while > 4% but < 30% of cells infected with each UK isolates were in the late apoptotic stage of death. There was a general drop in the proportions of necrotic cells 24 hrs post infection; these proportions ranged from 0.10% to 1.40% which is lower than the 0.65% to 4.70% range observed after 6 hrs.

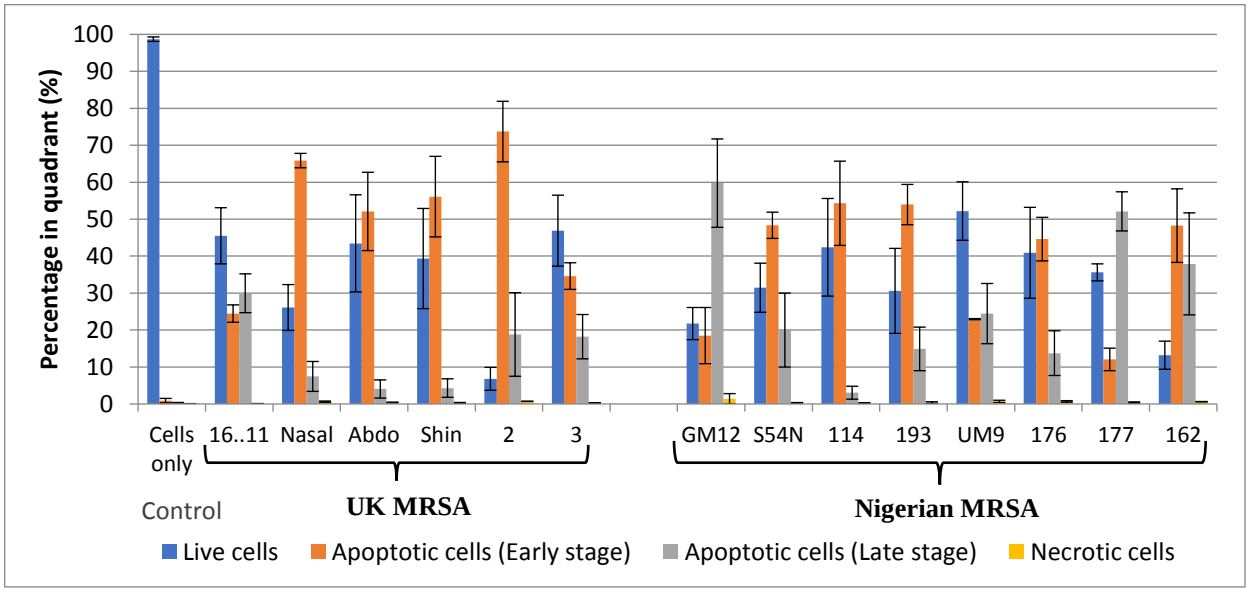


Figure 4.15: Distribution of apoptotic and necrotic A549 lung cells 24 hours following infection with live MRSA at MOI 10:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

Table 4.11: Proportional distribution of viable, apoptotic and necrotic cells 6 and 24 hours following infection of A549 epithelial cells with MRSA at MOI 10:1. Results are the mean of three independent experiments performed in duplicate.

MRSA Isolate (MOI 10:1)	Proportion of Viable cells (%)		Proportion of apoptotic cells % (Early stage)		Proportion of apoptotic cells % (Late stage)		Proportion of necrotic cells %	
	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs
UK								
16..11	5.65	45.50	78.17	24.45	5.63	29.95	2.50	0.10
Nasal	7.25	26.10	79.07	65.85	5.13	7.45	1.70	0.60
Abdo	10.65	43.45	76.40	52.10	6.27	4.05	1.90	0.40
Shin	6.80	39.35	75.97	56.10	7.53	4.30	1.95	0.30
2	6.35	6.80	78.50	73.70	6.07	18.80	1.85	0.70
3	16.80	46.90	79.37	34.60	3.27	18.20	0.65	0.25
Nigerian								
GM12	15.20	21.75	68.10	18.50	5.35	59.75	1.50	1.40
S54N	15.55	31.45	61.95	48.35	7.30	20.00	2.35	0.23
114	6.90	42.40	60.85	54.30	12.55	3.05	2.30	0.23
193	12.45	30.60	56.05	53.95	12.40	14.90	3.45	0.37
UM9	13.35	52.20	46.83	22.95	18.80	24.45	3.15	0.70
176	6.50	40.90	52.75	44.60	19.15	13.75	2.35	0.70
177	6.75	35.60	51.75	12.05	20.55	52.10	4.70	0.37
162	12.10	13.20	66.60	48.25	12.67	37.90	0.95	0.63

4.3.2.2.3. Distribution of cells six hours post-infection at MOI 100:1

At least 75% of A549 cells infected by each MRSA (UK and Nigeria) isolate at MOI 100:1 were in the early apoptotic death phase 6 hrs post-infection (Figure 4.16, Table 4.12). Though some of the cells were seen to be in the late apoptotic phase, the proportions were ≤ 15% post-infection with all the isolates. All proportions of necrotic A549 cell 6 hrs post-infection with all the isolates were seen to be ≤ 1.87%.

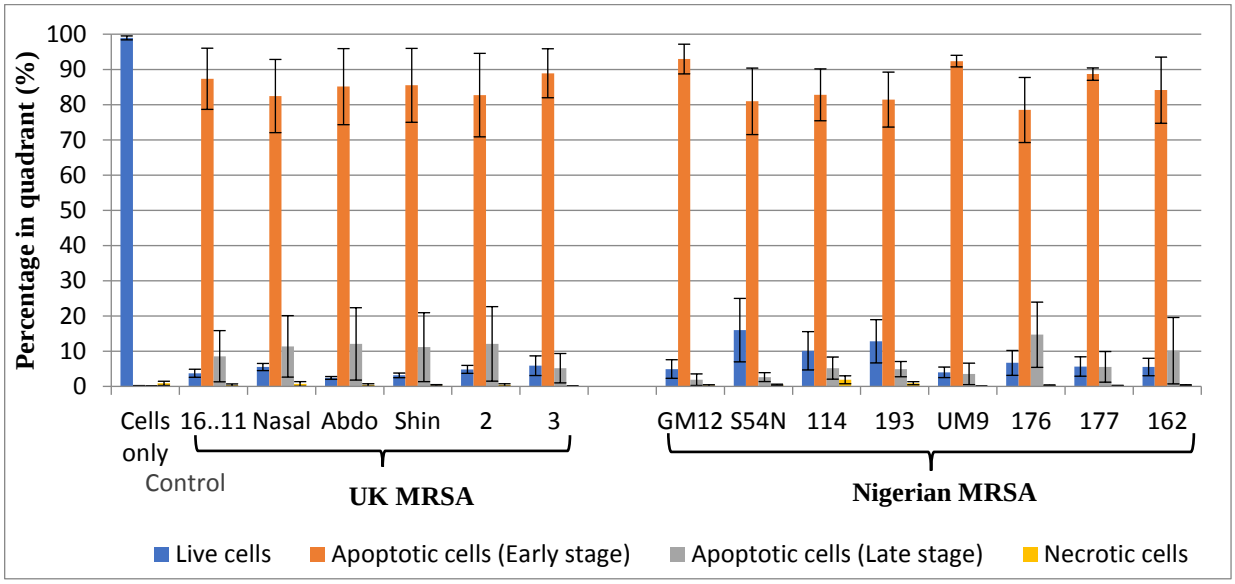


Figure 4.16: Distribution of apoptotic and necrotic A549 lung cells 6 hours following infection with live MRSA at MOI 100:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

4.3.2.2.4. Distribution of cells twenty-four hours post-infection at MOI 100:1

With the exception of UK isolate 193, an increase was observed in the proportions of viable cells 24 hrs post-infection (range from 4.85% - 58.85%) compared with 6 hrs post-infection (range from 4.00% - 15.97%) with all the MRSA isolates at MOI 100:1 (Table 4.12). Majority of A549 cells infected by MRSA at MOI 100:1 were in the apoptotic phase (early plus late = > 50%) except when isolate Nasal infected the cells) 24 hrs post-infection (Figure 4.17, Table 4.12). The proportions of necrotic A549 cells 24 hrs post-infection were seen to range from 0.00% to 1.35% (Figure 4.17, Table 4.12).

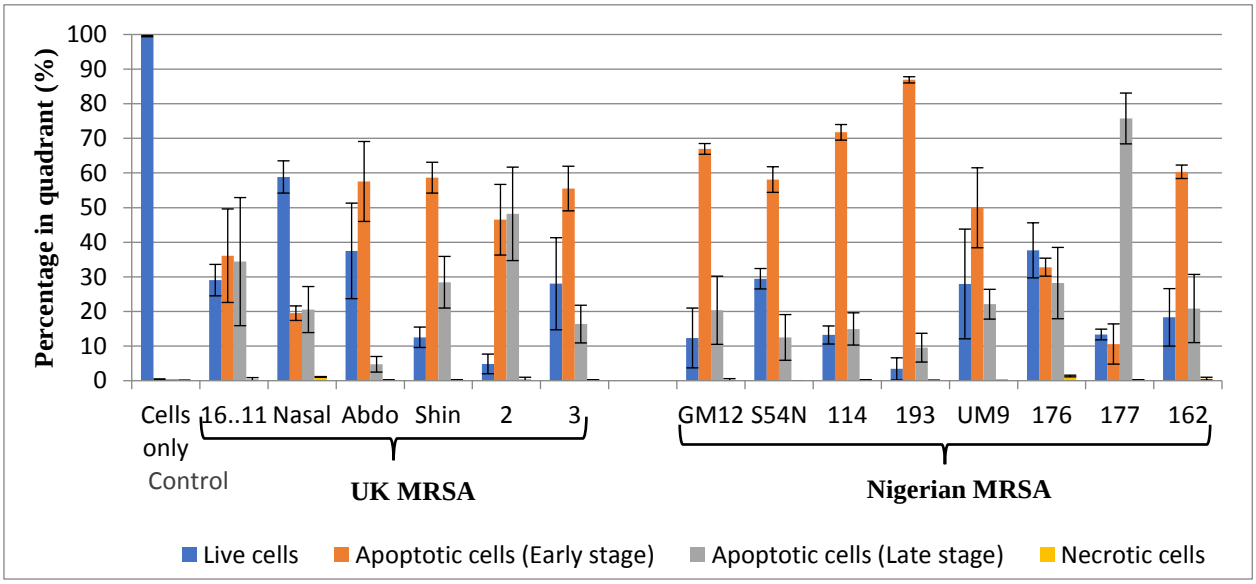


Figure 4.17: Distribution of apoptotic and necrotic A549 lung cells 24 hours following infection with live MRSA at MOI 100:1. Results are the mean of three independent experiments performed in duplicate; error bars represent standard error.

Table 4.12: Proportional distribution of viable, apoptotic and necrotic cells 6 and 24 hours following infection of A549 epithelial cells with MRSA at MOI 100:1. Results are the mean of three independent experiments performed in duplicate.

MRSA Isolate	Proportion of Viable cells (%)		Proportion of apoptotic cells % (Early stage)		Proportion of apoptotic cells % (Late stage)		Proportion of necrotic cells %	
	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs	After 6 hrs	After 24 hrs
UK								
16..11	3.73	29.05	87.33	36.10	8.57	34.40	0.37	0.45
Nasal	5.50	58.85	82.43	19.50	11.37	20.55	0.70	1.10
Abdo	2.40	37.50	85.10	57.55	12.07	4.75	0.40	0.15
Shin	3.13	12.55	85.47	58.65	11.13	28.45	0.30	0.30
2	4.83	4.85	82.70	46.50	12.07	48.20	0.40	0.50
3	5.87	28.00	88.90	55.50	5.17	16.35	0.07	0.20
Nigerian								
GM12	4.93	12.35	92.93	66.95	1.90	20.35	0.23	0.30
S54N	15.97	29.45	80.93	58.10	2.63	12.50	0.43	0.00
114	10.10	13.20	82.77	71.75	5.20	14.95	1.87	0.15
193	12.80	3.40	81.43	86.90	4.90	9.55	0.90	0.10
UM9	4.00	27.95	92.35	49.95	3.55	22.10	0.05	0.05
176	6.67	37.65	78.47	32.80	14.67	28.20	0.27	1.35
177	5.65	13.35	88.65	10.60	5.55	75.75	0.15	0.20
162	5.50	18.30	84.10	60.35	10.13	20.85	0.30	0.60

4.4. Discussion

Measuring leakage of cellular components from compromised cultured cells when membrane integrity is altered, and especially measurement of intracellular proteins (most often enzymes) in cell culture supernatants, is considered one of the most reliable methods for studying cell death (Mery *et al*., 2017). The lactate dehydrogenase (LDH) colorimetric assay, which measures the activity of LDH, was used to investigate the cytotoxic effects of MRSA on KB and A549 cell lines in this study. Lactate dehydrogenase has long been used as a marker of cell death in *in vitro* models (Allen *et al*., 1994; Mery *et al*., 2017) as it is released through the damaged membrane after cell death (Mery *et al*., 2017). Adenylate kinase and glucose-6-phosphate dehydrogenase are other cellular enzymes that can also be used as cell death markers, however, unlike LDH, loss in their activity can occur during cell death assays (Mery *et al*., 2017). Thus, cell death assays based on LDH activity are more effective than other enzyme-based cell death assays (Mery *et al*., 2017). Furthermore, LDH assays have the advantage of being relatively inexpensive (Kepp *et al*., 2011) and can easily be implemented in microtiter plates (Kepp *et al*., 2011; Mery *et al*., 2017) as done in this study.

Comparison of the means of cytotoxic effects of UK and Nigerian MRSA on KB epithelial cells (Section 4.3.1.1) showed that the Nigerian MRSA were more toxic on KB cells under all study conditions (MOIs 10:1 and 100:1; after 6 and 24 hrs; Live, Heat Killed and Conditioned Medium). The differences in toxicity were significant in all instances where live MRSA infected KB cells i.e. MOIs 10:1 and 100:1; after 6 and 24 hrs (Figures 4.1A, 4.2A, 4.3A and 4.4A; Tables 4.1 – 4.4). In the case of infection with heat killed MRSA, the differences in toxicity were significant only 6 hrs post-infection at MOIs 10:1 and 100:1 (Figures 4.1B and 4.3B; Table 4.1 and 4.3). Significant difference between the means of the cytotoxic effects of the CM of UK and Nigerian MRSA was seen only 24 hrs post-infection at MOI 100:1 (Figure 4.4C, Table 4.4). As was the case with the means, the results showed that medians of the cytotoxic effects of Nigerian MRSA

on KB cells were higher than those of UK MRSA under all the study conditions (MOIs 10:1 and 100:1; after 6 and 24 hrs; Live, Heat Killed and Conditioned Medium). Comparison of these medians revealed that the median cytotoxic effect of live Nigerian MRSA was only insignificantly higher than that of UK MRSA 6 hrs post-infection at MOI 10:1 (Table 4.1) but significantly higher in other instances (Tables 4.2 – 4.4). Medians of the cytotoxic effects of heat killed Nigerian MRSA were seen to be significantly higher than those of the UK MRSA in most instances (6 and 24 hrs post-infection at MOI 10:1; 6 hrs post-infection at MOI 100:1; Tables 4.1 – 4.3) but not 24 hrs post infection at MOI 100:1 (Table 4.4). Twenty four hrs post-infection at both MOI 10:1 and 100:1, the median of cytotoxic effects of the CM of Nigerian MRSA were seen to be significantly higher than those of the CM of UK MRSA (Table 4.2 and 4.4).

In the case of A549 cells (Section 4.3.1.2), means of the cytotoxic effects of the Nigerian isolates were seen to be higher than those of their UK counterparts under almost all study conditions i.e. MOIs 10:1 and 100:1; after 6 and 24 hrs; live, heat killed and conditioned medium (Tables 4.5, 4.6, 4.7 and 4.8); the exception being the mean of the toxic effects of the CM of UK MRSA on A549 cells at MOI 10:1 after 6 hrs (Table 4.5). Significant differences between the means of the toxic effects of UK and Nigerian MRSA on A549 cells were seen with live MRSA 6 hrs post-infection at both MOI 10:1 and 100:1 (Figure 4.5A and 4.7A; Tables 4.5 and 4.7) but not 24 hrs post-infection (Figures 4.6A and 4.8A; Tables 4.6 and 4.8). Similarly, significant differences were seen between the mean toxicities when heat killed MRSA infected A549 cell at MOI 10:1 and MOI 100:1 after 6 hrs (Figures 4.5B and 4.7B; Tables 4.5 and 4.7) but not after 24 hrs (Figures 4.6B and 4.8B; Tables 4.6 and 4.8). At no time were the means of the cytotoxic effects of the CM of Nigerian MRSA on A549 cells significantly higher than those of the CM of UK MRSA (Figures 4.5C, 4.6C, 4.7C and 4.8C; Tables 4.5 – 4.8). The results further showed that the medians of the cytotoxic effects of the CM of UK MRSA 6 hrs post-infection at both MOI 10:1 and 100:1 (Tables 4.5 and 4.7) as well as the medians of the cytotoxic effects of heat killed UK MRSA 24 hrs post-

infection at MOI 10:1 and 100:1 (Tables 4.6 and 4.8) were insignificantly higher than those of their Nigerian counterparts. Medians of the cytotoxic effects of live and heat killed Nigerian MRSA were however seen to be significantly higher than those of their UK counterparts 6 hrs post-infection at both MOI 10:1 and 100:1 (Tables 4.5 and 4.7).

In order to determine if there were skews (i.e. unevenness) between the toxic effects exerted by UK and Nigerian MRSA on KB and A549 cells, medians of the exerted cytotoxic effects exerted by UK and Nigerian MRSA on KB and A549 cells were determined and compared using the Mann-Whitney U test. Differences between the medians indicates that the values in the two data sets (the cytotoxic effects exerted by UK MRSA and those exerted by the Nigerian MRSA) are not similar; either the values in one set are higher or lower than those in the other set of data. Significant differences between the medians of the cytotoxic effects of UK and Nigerian MRSA (i.e. calculated U values $\leq$ Ucrit [10]) was observed under certain conditions in the course of this study. For example between the medians of the cytotoxic effects of heat killed MRSA on KB cells 6 hrs post-infection at MOI 10:1 (Table 4.1) as well as between the medians of the cytotoxic effects of live and heat killed on KB cells 6 hrs post-infection at MOI 10:1 (Table 4.5). In these examples the medians of the cytotoxic effects exerted by the Nigerian MRSA were higher than those of the UK MRSA; a look at the individual cytotoxic effects exerted by the MRSA shows the cytotoxic effects exerted by at least five Nigerian MRSA were higher than the cytotoxic effect exerted by the most toxic UK MRSA. In other words, the distribution of toxicities were skewed in favour of the Nigerian MRSA.

For the most part, findings from this study showed that there was correlation between the levels of significance of the means and medians of the cytotoxic effects of UK and Nigerian MRSA on the cell lines i.e. when the difference between means was significant, the difference between medians was also significant; when the difference between means was insignificant, the difference between

medians was also insignificant (Table 4.1 – 4.8). However, in several instances, disparities were observed between the levels of significance of the means and medians of the cytotoxic effects of UK and Nigerian MRSA on the cell lines. For instance although significance difference was observed between the means of the cytotoxic effects of live UK and Nigerian MRSA 6 hrs post-infection at MOI 10:1 (*P* value = 0.02817), no significant difference was seen between the medians of both sets of toxicities (calculated U value [11] > Ucrit [10]) (Table 4.1). This indicates that although the mean of the toxic effects of live Nigerian MRSA on KB cells was higher than that of live UK MRSA, the distribution of toxicities is not skewed in favour of the Nigerian MRSA.

In some other instances, differences between the means were insignificant, but differences between the medians were significant. This was seen when heat killed bacteria infected KB cells 24 hrs post-infection at MOI 10:1 (*P* value 0.08026; calculated U value [10] $\leq$ Ucrit [10]) (Table 4.2). Here, we see a skew in the distribution of the cytotoxic effects in favour of Nigerian MRSA; the cytotoxic effects of heat killed UK MRSA ranged from 1.37% - 6.75% while those of heat killed Nigerian MRSA ranged from 2.90% - 15.56%. Heat killed Nigerian isolates 'S54N' and '176' exerted greater toxic effects on KB cells (15.56% and 9.28% respectively) compared to heat killed UK isolate '3' which was the most toxic among the UK isolates. A look at the virulence factor profiles of these isolates revealed that the five pore forming toxins checked for in the course of this study (i.e. *hla, hlb, hlδ, hlγ* and PSMα) were present in Nigerian isolates 'S54N' and '176'; *hlb* was however not amplified from the genome of UK MRSA '3' (Section 2.3.3, Table 2.3).

Another instance was also seen in the cytotoxic effect of MRSA CM on KB cells 24 hrs post-infection at MOI 100:1 (*P* value 0.0818; calculated U value [7] < Ucrit [10]) (Table 4.2). A skew was also seen in the distribution of cytotoxic effects in favour of the Nigerian MRSA as the cytotoxic effects of the CM of UK MRSA on KB cells was seen to be 5.07% - 27.44% while those of the CM of Nigerian MRSA ranged from 7.03% - 45.95% (Table 4.2). This time around,

cytotoxic effects of the CM of Nigerian isolates 'GM12' (45.95%) and 'UM9' (34.09%) were higher than those of the CM of UK isolate 'Nasal' (27.77%) which exerted the greatest cytotoxic effect on KB cells among the UK isolates (Table 4.2). The virulence factor profiles of the isolates showed that genes encoding the five pore forming toxins checked for in the course of this study (i.e. *hla, hlb, hlδ, hlγ* and PSMα) were amplified from the genomes of both Nigerian isolates 'GM12' and 'UM9' (Chapter 2, Table 2.3). On the other hand, only the genes encoding *hla, hlδ, hlγ* and PSMα were amplified from the genome of UK isolate 'Nasal' (Section 2.3.3, Table 2.3).

Although both the differences between the means as well as between the medians of the cytotoxic effects exerted by the CM of UK and Nigerian on KB cells were insignificant 6 hrs post-infection at MOI 10:1 (*P* value = 0.09485; calculated U value [20] > Ucrit [10]), the toxic effects of the CM of Nigerian isolates '177' (51.12%) and '162' (52.80%) were more than double that of the cytotoxic effect of the CM of UK isolate '2' (18.38%) which was the most toxic among the UK isolates (Table 4.1). All five pore forming toxins checked for in this study i.e. *hla, hlb, hlδ, hlγ* and PSMα, were seen to be present in both Nigerian isolate 'GM12' and UK isolate '2' (Section 2.3.3, Table 2.3). Nigerian isolate '162', whose CM was the most toxic on KB cells in this instance, was seen to bear the *hlb, hlδ* and PSMα pore forming toxins, but not *hla,* and *hlγ* (Section 2.3.3, Table 2.3). Except for the type of capsular polysaccharides they bear, results presented in Table 2.3, showed that the virulence factor profile of UK isolate '16/11' (whose CM exerted a 4.48% toxicity on KB cells) was identical to that of Nigerian isolate '177' (whose CM exerted a 51.12% toxicity on KB cells).

Agr-regulated factors are essential for *S. aureus* mediated cytotoxicity (Schnaith *et al*., 2007); and among these *agr*-regulated factors, the α-toxin is known to be an important inducer of toxicity (Prince *et al*., 2012; Loffler *et al*., 2014). Results from chapter two showed that all (100%) the UK MRSA and 5 (62.5%) of Nigerian MRSA used in the cytotoxicity experiment bear the α-toxin gene

(Table 2.3, Table E.5 (Appendix E)). Nevertheless, the results showed that absence of the α-toxin gene did not in any way diminish the toxicity/virulence potential of Nigerian isolates '114', '193' and '162' which did not bear the α-toxin gene (Tables 4.1 – 4.8). It can be seen that these Nigerian isolates were even more toxic on KB and A549 cells than UK MRSA in the different instances of toxicity i.e. MOIs 10:1 and 100:1; 6 and 24 hrs post-infection; live , heat killed and bacterial CM.

Studies like those by Bayles *et al*., 1998, Shompole *et al*., 2003, Schnaith *et al*., 2007, Jarry *et al*., 2008 and Strobel *et al*., 2016, have previously shown that cytotoxic strains of *S. aureus* are able to escape the phagolysosomal vesicles and ultimately lead to host cell death. Moreover, Giese *et al*., 2009 demonstrated in their study that the alpha toxin alone is not sufficient to mediate phagolysosomal escape in upper-airway epithelial cells. Furthermore, Bantel *et al*., 2001, previously showed that *S. aureus*–induced cytotoxicity can be mediated by α-toxin and does not require bacterial internalisation. In line with these, it can be said from the findings of this study that (i) the alpha toxin alone is not sufficient for phagolysosomal escape, of internalised bacteria, that ultimately led to host cell death, and (ii) the alpha toxin present in the bacterial CM is not the only virulence factor responsible for the observed cytotoxic effects of MRSA CM on the cell lines. Thus, though the alpha toxin may have played a role in the observed cytotoxic effects of the MRSA isolates on the cell lines in this study, the results clearly show that cytotoxicity cannot be wholly ascribed to this toxin and that differences in the observed cytotoxicity induced by the UK and Nigerian MRSA isolates cannot be based on the presence or absence of the alpha toxin.

Results from the characterisation of the isolates (Chapter 2) showed that the sphingomyelinase β-toxin (*hlb* gene) is present in all the Nigerian isolates but in only 50% of the UK isolates (Section 2.3.3, Table 2.3). Although they did not bear the *hlb* gene, there were several instances where UK isolates 'Nasal', 'Abdo' and '3', exerted greater toxicity on KB and A549 cells than their Nigerian counterparts that bear the *hlb* gene. For example, 24 hrs post-infection at MOI 100:1, live UK

isolate ‘Nasal’ was more toxic on KB cells (40.13%) than Nigerian isolates ‘114’ (28.53%), ‘176’ (31.21%) and ‘177’ (40.09%) (Figure 4.4A, Table 4.4). Under the same conditions, live UK isolate ‘3’, was more toxic on KB cells (31.37%) than its Nigerian counterparts ‘114’ (28.53%) and ‘176’ (31.21%) (Figure 4.4A, Table 4.4). In addition, it can be seen that 24 hrs post-infection at MOI 10:1, live UK isolates ‘Nasal’ and ‘Abdo’ were more toxic on A549 cells (13.70% and 13.54% respectively) than Nigerian isolates ‘114’ (8.78%), ‘193’ (2.29%), ‘UM9’ (10.16%) and ‘176’ (6.66%) (Figure 4.6A, Table 4.6).

Similarly, 100% of the Nigerian isolates and only 66.67% of the UK isolates bear the γ-haemolysin (*hlγ*) (Section 2.3.3, Table 2.3) However, there are instances where UK isolates ‘Abdo’ and ‘Shin’ which both lack the *hlγ* gene exert greater toxicity on host cells than their Nigerian counterparts that bear the *hlγ* gene. For example, 6 hours post-infection at MOI 10:1, live UK isolates ‘Abdo and ‘Shin’ are more toxic (2.00% and 1.88% respectively) than Nigerian isolates ‘S54N’ (1.75%) and ‘114’ (0.36%) (Figure 4.1A; Table 4.1). Also, 24 hrs post-infection at MOI 10:1, live UK isolate ‘Abdo’ was more toxic A549 cells (13.54%) than Nigerian isolates ‘114’ (8.78%), ‘193’ (2.29%), ‘UM9’ (10.16%) and ‘176’ (6.66%) (Figure 4.6A, Table 4.6). Six hours post-infection at MOI 10:1, the CM of UK isolates ‘Abdo and ‘Shin’ were more toxic (3.61% and 2.06% respectively) than the CM of Nigerian isolates ‘114’ (0.00%) and ‘176’ (0.29%) (Figure 4.1C; Table 4.1). Furthermore, 6 hours post-infection at MOI 100:1, the CM of UK isolates ‘Abdo and ‘Shin’ were seen to be more toxic (3.98% and 5.28% respectively) than the CM of Nigerian isolates ‘114’ (2.94%) and ‘176’ (2.45%) (Figure 4.3C, Table 4.3).

Nigerian isolates ‘114’ and ‘162’ were also seen to lack the gene that encodes *δ* toxin in addition to lacking the α-toxin gene (Section 2.3.3, Table 2.3). Notwithstanding, these two isolates exerted varying degrees of toxiciy on the cell lines, sometimes even higher than the toxic effects exerted by the isolates that bear both the *hla* and *hlδ* genes. For example, 24 hrs post-infection at MOI

10:1, live Nigerian isolates '114' and '162' were more toxic on KB cells (22.56% and 62.49% respectively) than all the UK MRSA (Figure 4.2A, Table 4.2) which bore both the *hla* and *hlδ* genes (Table 2.3). The same was also the case 6 hrs post-infection at MOI 10:1, when live Nigerian isolates '114' and '162' were more toxic on A549 cells (4.66% and 5.08% respectively) than all the UK MRSA (Figure 4.5A, Table 4.5).

These findings point to the likelihood that the beta, gamma and delta toxins played prominent roles in the cytotoxic effects exerted by the UK and Nigerian MRSA isolates on the KB and A549 cell lines; these toxins could also be the basis for the observed differences in toxicities. In their work to describe the activity of virulence factors mediating phagosomal escape in infected epithelial and endothelial cell lines, Giese *et al*., 2011 found that synergistic activity of the staphylococcal δ-toxin and the sphingomyelinase β-toxin enable the phagosomal escape of *S. aureus*. Based on this, it can be hypothesised that, as demonstrated by Giese *et al*., 2011, synergistic activity between toxins, other than the alpha toxin, played crucial roles in the observed differences in cytotoxic effects.

The ability of *S. aureus* to leave the phagolysosomes is also linked to the PSMα gene (Strobel *et al*., 2016). This was demonstrated in the study by Grosz *et al*., 2014, where it was seen that phagosomal escape of clinically relevant *S. aureus* strains i.e. *S. aureus* LAC, MW2, and 6850 strains, was mediated by a common PSMα-dependent mechanism despite the distant genetic backgrounds of the strains. All the MRSA isolates involved in this study (both UK and Nigerian) were seen to bear the PSMα gene (Section 2.3.3, Table 2.3). Results from the cytotoxicity assays (Tables 4.1 – 4.8) showed that live UK and Nigerian MRSA exerted varying levels of toxicity on both KB and A549 lung epithelial cells. Thus, the PSMα toxin may be responsible for the cytotoxic effects exerted by all the live UK and Nigerian MRSA on the cell lines regardless of their genetic

backgrounds. Nevertheless, the ability to produce PSMα alone does not explain why some isolates were more toxic on cells lines than others.

As observed in the present study, the cell-free CM of both the UK and Nigerian MRSA isolates exerted toxic effects, in varying degrees, on the mammalian cell lines (Figures 4.1C, 4.2C, 4.3C, 4.4C, 4.5C, 4.6C, 4.7C and 4.8C). However, the fact that there were no significant differences between the means of the toxic effects of the cell-free CM of both the UK and Nigerian isolates on the mammalian cell lines (Tables 4.1 – 4.3 and 4.5 – 4.8) indicates that both sets of MRSA isolates secrete similar virulence factors for the most part. In addition to live bacteria, invasion of host cells is equally effective with killed bacteria (Strobel *et al*., 2016). However, as expected, HK MRSA were seen to exert the least toxic effect (particularly after 24 hrs) on both KB and A549 lung cell lines compared to live MRSA and MRSA CM (Figures 4.2B, 4.4B, 4.6B, 4.8B), an indication that heat indeed denatures bacterial proteins thereby rendering the bacteria ineffective.

Results obtained show that the mean cytotoxic effects of the CM (prepared by inoculating $10^6/10^7$ CFU bacteria in 5 ml EMEM/DMEM) of the both the UK and Nigerian MRSA isolates on the mammalian cells (KB and A549 cell) were higher than those exerted by live MRSA after 6 hrs (Tables 4.1, 4.3, 4.5 and 4.7). It was only at MOI 10:1 24 hrs post-infection that the mean cytotoxic effect of the CM (prepared by inoculating 10^6 CFU bacteria in 5 ml DMEM) of UK MRSA was higher than the mean cytotoxic effect of live UK MRSA on A549 cells (Table 4.6). Otherwise the mean cytotoxic effects of live MRSA (UK and Nigerian) on the mammalian cell (KB and A549) were higher than those exerted by the CM of the isolates after 24 hrs (Table 4.2, 4.4, 4.6 and 4.8). The CM has previously supported bacterial growth and thus contains numerous secreted factors endogenous to the particular strain of bacteria. Bear in mind that the MRSA isolates were inoculated in media and incubated overnight (18 hrs) to obtain the CM. In other words, the factors were secreted into the media over an 18-hr period. Thus, it is unsurprising that these CM exerted greater toxic effects on the mammalian cells than the live MRSA 6 hrs post-infection because the

live MRSA have had only 6 hrs to secrete metabolites into the medium. Meaning there were less factors in the medium with live MRSA compared to the culture containing the CM. However, after 24 hrs, the live MRSA have had time to secrete more factors, thus the increase in toxic effect. These combined with the surface bound factors may have played a role in the observed differences in the toxic effects of live MRSA and bacterial CM on the mammalian cells.

A comparison of results obtained from the interaction assays and results from the cytotoxicity assays indicate that the rate at which MRSA invaded the mammalian cell lines could have played a role in the cytotoxic effect of live MRSA on mammalian cells. This is because similar to results from the invasion assays which showed that on average the UK and Nigerian MRSA invaded the KB epithelial cells more than they did the A549 lung cells at both MOIs (Sections 3.3.3 and 3.3.4, Tables E.5A A (Appendix E) and E.6A A (Appendix E)), means of the cytotoxic effects of live UK and Nigerian MRSA on KB cells after 24 hrs were higher than means of the cytotoxic effects on A549 cells under the same conditions (Tables E.5A B (Appendix E) and E.6A B (Appendix E)). However, these differences in toxicities after 24 hrs were not significant.

Like other enzyme-based cell death assays, the LDH cytotoxicity assay is limited by the fact that it is unable to discriminate between different cell death modes, and detection may be aggravated by morphologic changes in dying cells (Mery *et al*., 2017). Furthermore, the activity of these enzymes may be affected by physicochemical parameters (for example, changes in the pH of the culture medium) (Kepp *et al*., 2011; Mery *et al*., 2017). Therefore, in order to discriminate between different cell death modes that were involved in the observed cytotoxic effects of MRSA on KB and A549 cell lines, apoptosis/necrosis assays based on cell membrane changes and phosphatidylserine (PS) exposure were carried out in the course of this study. The exposure of phospholipid phosphatidylserine on the cell membrane in response to proapoptotic stimuli represents a major feature of apoptosis in healthy cells (Mery *et al*., 2017). Therefore, a PS-binding protein such as AnnexinV is experimentally used to detect PS exposure (Mery *et al*., 2017). When

the plasma membrane is permeabilised, Annexin V binds intracellular phosphatidylserine, implying that this staining is incompatible with sample permeabilisation, and that necrotic cells, irrespective of their origin, will stain positively for Annexin V (Kepp *et al*., 2011). Therefore, to distinguish apoptotic cells (which are Annexin V-positive but with intact plasma membranes) from non-apoptotic cells, Annexin V staining is usually combined with cell-impermeable dyes, such as propidium iodide (Kepp *et al*., 2011; Mery *et al*., 2017). The Zombie dye was used in this study. Although it is a sensitive and rapid method to assess apoptosis, PS exposure can also occur in cell death–unrelated conditions, especially when T lymphocytes are activated (Kepp *et al*., 2011; Mery *et al*., 2017). Furthermore, some cell types do not expose phosphatidylserine during apoptosis, and this is an aberration that has been linked to defects in the phosphatidylserine-exposing enzyme phospholipid scramblase 1 (Kepp *et al*., 2011).

Results from the flow cytometry experiments showed that proportions of viable cells (KB and A549) six hrs post-infection with the MRSA isolates (both UK and Nigerian at MOIs 10:1 and 100:1) were less that than those at 24 hrs post-infection (Tables 4.9 – 4.12). This finding is possibly due to the continued proliferation of uninfected and even possibly infected cells. As previously demonstrated in several studies, when virulent *S. aureus* strains are added to host cells in tissue culture, host cell death occurs via mechanisms that have been mainly identified as apoptotic (Bayles *et al*., 1998; Menzies and Kourteva, 1998; Wesson *et al*., 1998,; Tucker *et al*., 2000; Haslinger *et al*., 2003). In line with this, findings from this study show that all six UK and eight Nigerian MRSA predominantly induced apoptosis (late and early) in both KB and A549 cells after 6 and 24 hrs following infection at MOIs 10:1 and 100:1 (Tables 4.9 – 4.12). These findings further support the observation made earlier, i.e. that differences in the observed cytotoxicity induced by the UK and Nigerian MRSA isolates cannot be based on the presence or absence of the alpha toxin. This is because three of the eight Nigerian isolates (114, 193, 162) did not bear the alpha toxin gene (Section 2.3.3, Table 2.3), yet results from the flow cytometry analysis show that all six UK

and eight Nigerian MRSA induced both apoptosis (predominantly) and necrosis (minimally) (Tables 4.9 – 4.12).

In conclusion, the Nigerian MRSA were seen to exert higher toxic effects on KB and A549 cells than UK MRSA; however, the means of the toxic effects of Nigerian MRSA on KB and A549 cells were significantly higher than those of UK MRSA mostly when live MRSA infected host cells. Although virulence factors borne by *S. aureus* are known to mediate the cytotoxic effect, the virulence factor(s) responsible for the observed differences between the cytotoxic effects of UK and Nigerian MRSA on host cells could not be ascertained based on the virulence factor profiling (Section 2.3.3, Table 2.3) done in the course of this study.

Chapter Five

Protein Analysis

5.1. Introduction

Sequencing an organism's DNA complement reveals its genetic makeup; however, proteins are the component that functionally govern most cellular processes (Curreem *et al*., 2012; Mesri *et al*., 2014). The constituent proteins of a cell carry out their functions at specific times and locations in the cell, in physical or functional association with other proteins or biomolecules (Aebersold and Mann, 2016). The genome contains a set number of genes that encode proteins or functional RNA molecules, e.g. ribosomal RNA (rRNA), transfer RNA (tRNA) or non-coding RNA (ncRNA) (Curreem *et al*., 2012). The genome sequence of a bacterial species or an isolate gives insight into its biochemistry and cellular physiology, and an understanding of how it may adapt to its specific ecological niches (Curreem *et al*., 2012). It also allows for study of the evolutionary history and genetic relationships of a bacterial species to other organisms (Curreem *et al*., 2012). Furthermore, genomic information has led to massive advancements in drug and vaccine discovery, molecular epidemiology, diagnostic bacteriology and knowledge of antimicrobial resistance mechanisms (Curreem *et al*., 2012). Regardless of these, gene sequence data contain insufficient information to understand the function of the gene products (Kellner, 2000). Neither DNA nor mRNA encodes the arrangement for, e.g., a signalling pathway or for a metabolic cascade (Kellner, 2000). This lack of correlation between gene expression and protein function can be attributed, but not limited, to (i) post-transcriptional splicing and recombination of RNA leading to various protein products i.e. after synthesis on ribosomes, proteins are cut to eliminate initiation, transit, signal sequences, as well as simple chemical groups or complex molecules that are attached; (ii) co- and post-translational events such as the addition of different functional groups to a protein (e.g., sugars or phosphate), enzymatic cleavage of premature proteins to become active and intra- or intermolecular interactions leading to formation of a functional oligomer or a protein complex (Kellner, 2000; Cho, 2007; Kumar *et al*., 2016). Thus, several isoforms or proteoforms with distinct structural and functional attributes may originate from a given gene (Kumar *et al*., 2016).

As such, DNA sequence analysis does not predict the active form of a protein and RNA quantitation does not always reflect the corresponding protein levels (Cho, 2007). A good understanding of gene expression patterns specifically associated with a given biological state is fundamental to understanding cellular processes and diseases (Kumar *et al*., 2016).

A proteome can be defined as the overall protein content of a cell that is characterised with regard to their localisation, interactions, post-translational modifications and turnover, at a particular time (Aslam *et al*., 2017). The extensive proteome network of a cell adapts dynamically to external or internal (genetic) perturbations and thereby defines the cell's functional state and determines its phenotypes (Aebersold and Mann, 2016). While a genome remains unchanged to a large extent, the proteins in any particular cell change dramatically as genes are turned on or off in response to the environment (Cho, 2007). Thus, the proteome is like a photographic snapshot of the protein expression at a particular moment and under specific conditions (Lopez, 2007). As such, it can be said that there is one particular genome for every given organism or cell, but there is an infinite number of proteomes when referring to protein expression (Lopez, 2007). A comprehensive description of the proteome of an organism therefore provides a catalogue of all proteins encoded by the genome as well as data on protein expression under defined conditions (Lopez, 2007). Proteome analysis provides the complete depiction of structural and functional information of cell as well as the response mechanism of cell against various types of stress and drugs using single or multiple proteomics techniques (Curreem *et al*., 2012).

Although genotypic information is valuable in identifying an organism and determining how it is related to others, methods that probe an organism's phenotypic properties remain critical for understanding the physiological and functional activities of an organism at the protein level (Emerson *et al*., 2008). Proteomics bridges the gap between genomic information and functional proteins and translates this information (Mesri *et al*., 2014). Proteomics refers to the characterisation of proteome, including expression patterns, macromolecular protein structures,

biological functions, spatiotemporal intracellular distributions, stabilities and turnover rates, protein-protein interactions and modifications of proteins at any stage (Curreem *et al*., 2012; Aslam *et al*., 2017).

The advent of proteomics tools, that allow rapid interrogation of biomolecules produced by an organism, offers an excellent complement to classical microbiological and genomics-based techniques for bacterial classification, identification, and phenotypic characterisation (Emerson *et al*., 2008). Examination of protein profiles by proteomic analysis has become an essential tool for studying the basic mechanisms of bacterial resistance and virulence (Perez-Llarena and Bou, 2016). This has led to a better understanding of the biology of pathogens (e.g. global post-translational protein modifications, subcellular protein location, and protein turnover rates) that cannot be investigated by reductionist or even genomic studies (Perez-Llarena and Bou, 2016). Moreover, proteomics is recognised for its potential to describe cell/tissue differentiation and discovery of diagnostic markers for disease (Manzoni *et al*., 2016).

As previously discussed in section 4.4, results obtained from the cytotoxicity assays (section 4.3.1) indicated that both UK and Nigerian MRSA isolates secreted similar virulence factors, because there were no significant differences between the means of the toxic effects of the cell-free CM of both the UK and Nigerian isolates on KB and A549 cell lines (Tables 4.1 – 4.3, Tables 4.5 – 4.8). Consequently, in a bid to determine the reason for the observed differences in the cytotoxic effects of the UK and Nigerian MRSA isolates, the intracellular proteins extracted from MRSA isolates were subjected to two-dimensional electrophoresis (2-DE) and compared.

5.2. Materials and Methods

5.2.1. Bacterial strains and culture

The isolates used for this study were selected based on clonal distribution. UK isolates 16/11 and Abdo were selected because they were un-typeable based on the clonal typing scheme used in this study. UK isolates Nasal and Shin were selected because they belong to the CC22 and CC30 lineages respectively. Nigerian isolates GM12 and S54N were included in this study because they were un-typeable while isolates 114 and 193 were selected on the basis of their belonging to the ST239 lineage. All eight isolates (four UK and four Nigerian) were grown and maintained as described in section 2.2.1 (Chapter Two). Optical densities of bacterial broth cultures were measured in a spectrophotometer as described in section 2.2.1 (Chapter Two) i.e. OD_{595} 0.22 = ~10^8 CFU/ml.

5.2.2. Intracellular protein extraction

In order to obtain the proteins not secreted by bacteria (i.e. intracellular proteins), the MRSA isolates were first plated onto CLED agar and incubated under aerobic conditions at 37°C overnight. Afterward, a loopful of colonies from these plates were inoculated into LB broth and incubated for 6 hrs (exponential phase cultures). The resulting broth cultures were centrifuged for 10 mins at 5000 rpm/3,354 x g following which supernatants were separated from the pellets. The pellets were washed twice with PBS following which they were re-suspended in 50 µl sample buffer [0.06M Tris, 2.5% glycerol, 0.5% sodium dodecyl sulphate (SDS), 1.25% beta-mercaptoethanol, bromophenol blue 0.001% (pH=6.8)]. Proteins were then denatured by placing in boiling water for 5 mins.

5.2.3. Bradford protein assay

The concentrations of proteins extracted from each isolate were determined using Bradford assays. Protein extracted from the isolates were compared with five different standard protein

concentrations of bovine serum albumin (BSA) dissolved in PBS i.e. 2 mg/ml, 1 mg/ml, 0.5 mg/ml, 0.25 mg/ml, and 0.125 mg/ml. Bradford reagent was added to each protein sample after which each sample was incubated at room temperature for 10 mins. The spectrophotometer was set to 595 nm and the absorbance of standards, the blank, and the proteins of unknown concentration were measured.

5.2.4. Two-dimensional (2-D) gel electrophoresis

5.2.4.1. First-dimensional gel isoelecric focusing (IEF)

Isoelecric focusing is an electrophoretic method that separates proteins according to their isoelectric points (pI) (Berkelman and Stenstedt, 2004; Slibinskas *et al*., 2013) i.e. the specific pH at which the net charge of the protein is zero (Berkelman and Stenstedt , 2004; Maurer and Kuschinsky, 2007). Majority of *S. aureus* proteins are clustered between pH 4 and 6 (Assafi, 2016). Therefore, to allow for better separation of proteins, immobilised pH gradient (IPG) strips of pH 4-7 were used for this study. Immobiline 11 cm dry-gel strips with a pH range of 4 – 7 (GE Healthcare Bio-Sciences AB) were rehydrated in a dry-strip re-swelling tray. Each dried strip was rehydrated for 24 hrs at room temperature in 300 µl of a rehydration buffer (7 M urea, 2 M thiourea, 4 % CHAPS, protease inhibitor cocktail 1X, 20 mM DTT, 1 % ampholyte, Bromophenol Blue 0,1 %, 0.05 % SDS) containing 60 µg of the protein sample. The IPG strip was covered with ~3 ml immobiline dry-strip cover fluid, PlusOne (GE Healthcare Bio-Sciences AB). After rehydration, the IPG strip was gently rinsed with distilled water to remove any crystallised urea. The strip was then subjected to Isoelectric focusing (IEF) by using the IPG-Phor3 control software. The IPG-Phor manifold was covered with 108 ml of Immobiline PlusOne dry-strip cover fluid and the rehydrated strip was then placed in individual lanes of the Ettan IPG strip holder (with the positive end towards the anode end of the main fold). Paper electrode wicks were placed between the IPG

strips and the electrodes. The lid was closed, and the IPG-Phor program was run according to the following stepwise program;

Step 1: 500 V 6 hrs

Step 2: 1000 V 7 hrs 4 mins

Step 3: 6000 V 2 hrs

Step 4: 8000 V 2 hrs 22 mins

Step 5: 200 V 1 hr

At the end of the programme, the IPG strips were placed in Petri dishes, rinsed briefly with deionised water, labelled, and stored at -80^{o}C for later use.

5.2.4.2. Equilibration of the IPG strips

Before the second-dimension gel run, the IPG strips comprising isoelectrically focused proteins were equilibrated and reduced. Each IPG strip was incubated at room temperature for 15 mins with 10 ml of equilibration buffer (6M urea, 0.375M pH8.8 Tris-HCl, 4% SDS, 20% glycerol, H_2O, and phenol red) containing 100 mg of Dithiothreitol (DTT). Afterward, the strip was incubated for 15 mins at room temperature with 10 ml of another equilibration buffer containing 250 mg of iodo-acetamide. The IPG strip was rinsed with 1X electrophoresis buffer (3 g Tris, 15 g glycine, 10 ml 10% SDS, and distilled water up to 1 L) before being placed on the gel.

5.2.4.3. Assembly and running of second-dimensional gel

In the second dimension, proteins were separated according to their molecular weights (mws) independent of the intrinsic electrical charge of the proteins (Berkelman and Stenstedt, 1998; Maurer and Kuschinsky, 2007). The equilibrated IPG strip was placed into the well of a 12% SDS-PAGE gel and sealed with agarose sealing solution, avoiding any air bubbles. Electrophoresis was carried out first at 50 V for 30 mins and then at 100 V for approximately 2 – 3 hrs or until the

bromo-phenol blue dye front had reached the lower end. Then, the gel was fixed with a gel-fixing solution (50% ethanol, 12% acetic acid, and 0.05% formalin).

5.2.4.4. Protein visualisation

For visualisation of protein spots a modified silver staining protocol was used (Yan *et al.*, 2000; Gromova and Celis, 2006). The gel was washed with 20% methanol three times and then sensitised with 0.02% sodium thiosulfate and washed twice with distilled water. Staining was done with 0.2% silver nitrate solution for 20 mins, followed by a careful wash twice with distilled water for a maximum of 1 min each time. The gel was developed with 6% sodium carbonate and 0.0004% sodium thiosulfate for 3 mins or until spots appeared. Finally, the reaction was stopped by adding 12% acetic acid and shaking it for 10 mins. The gel was stored in 5% acetic acid at 4°C.

5.2.4.5. Gel image capture and spot analysis

The gels were scanned using the Epson scanner image III with LabScan 6.0 software. First, the scanner was calibrated and configured to use the transparent setting to 300 dpi with a blue filter. The scanner surface was cleaned with 70% ethanol and some purite water that was poured on the surface. The gel was placed directly onto the scanner preview and air bubbles were smoothed out. Then, the scanning area of the gel was selected and scanned. Gel images were saved as MEL and TIF files. Gel scanned images were characterised using the Progenesis SameSpots software 3.3.3420.25059 (Nonlinear Dynamics, Newcastle upon Tyne, UK).

5.2.5. Protein identification

The ExPASy Tagldent tool (https://web.expasy.org/tagident/) was used to predict the identities of protein spots within specified pI and mw regions.

5.3. Results

Intracellular proteins extracted from all the isolates involved in this study were subjected to 2-DE analysis. Proteins were separated in the first dimension by isoelectric focusing on pH 4-7 IPG strips (11cm). The isoelectrically focused proteins were then resolved by second-dimension SDS-PAGE which separated the proteins according to their molecular weights. The protein spot pattern of silver stained gels for the four UK and four Nigerian isolates were analysed as 2 separate groups, i.e. the UK group and Nigerian group, using the Progenesis SameSpots software. Fifty well-resolved protein spots, generated after silver staining of the gels, were randomly selected from the 7904 detected spots by the Progenesis SameSpots software for the UK isolates; these selected spots have molecular weights ranging from 15 to 150 kDa and isoelectric points (pIs) (i.e. the pH at which a protein has no net charge) ranging from 4.4 to 6.5 (Tables 5.1 - 5.2, Figures 5.1 – 5.4,). Also, 50 well resolved proteins spots, generated after silver staining of the gels, were randomly selected from the 3251 detected spots by the Progenesis SameSpots software for the Nigerian isolates; these selected spots were seen to have molecular weights ranging from 16 to 51 kDa, and pIs ranging from 4.6 to 6.8 (Figures 5.6 – 5.9, Table 5.4). Protein spots were selected based on manual/visual inspection of both the 2D montage (Figures 5.1 – 5.4 and 5.6 - 5.9) and 3D montage (exemplified in Figures 5.5 and 5.10) views of spots detected by the Progenesis software.

5.3.1. UK group

In the UK group, only six of the 50 selected spots were found in isolate Shin (Table 5.1). While isolates 16/11 and Abdo were seen to have 18 spots each, 27 spots were detected in isolate Nasal (Table 5.1). While isolate Abdo had eight spots in common with isolate Nasal (i.e. 1912, 2171, 2624, 2712, 3024, 3085, 4730 and 5295), isolate 16/11 had seven spots in common with isolate Nasal (i.e. 2459, 2503, 2712, 2821, 3509, 3608 and 5186) (Table 5.1). Only spots 2443 2599 and 2712 were common to isolates 16/11 and Abdo (Table 5.1). Furthermore, only spot 2712 was common to isolates 16/11, Abdo and Nasal. Isolate Shin shared spots 4113, 5295 and 7242 in common with isolate Nasal; protein spot 5295 was common to isolates Shin, Nasal and Abdo (Table 5.1). Spot 623 which had the highest molecular weight among the 50 protein spots selected for the UK group (i.e. 150 kDa; Table 5.2) was seen to be present only in isolate Nasal (Table 5.1). Proteins with the lowest molecular weight i.e. 15 kDa were absent from isolate Abdo but were present in isolates 16/11 (spots 7465, 7883), Nasal (7194, 7207, 7242, 7420) and Shin (7242, 7876).

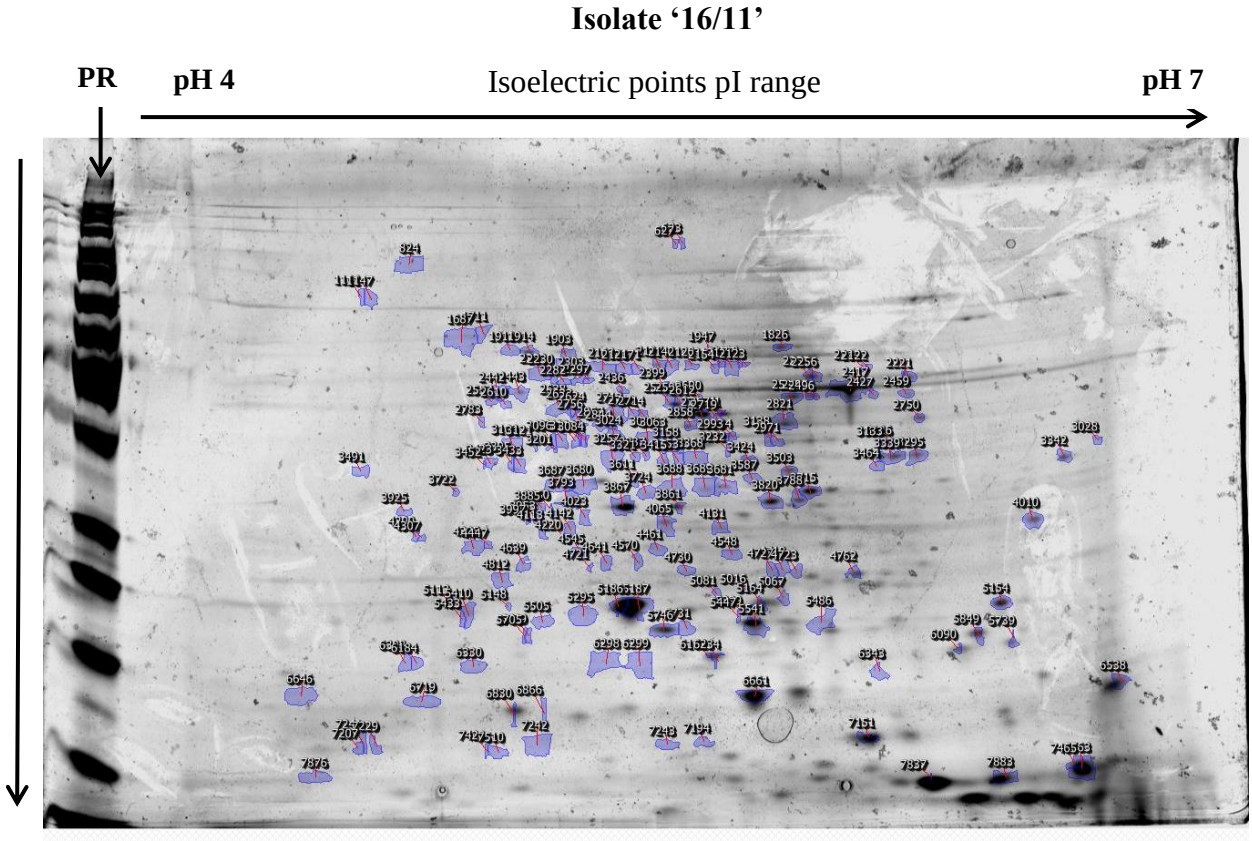
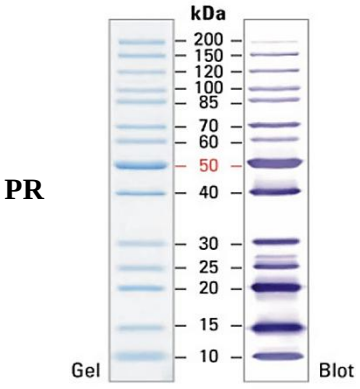


Figure 5.1: Silver stained 2-D gel showing intracellular proteins from UK isolate '16/11'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

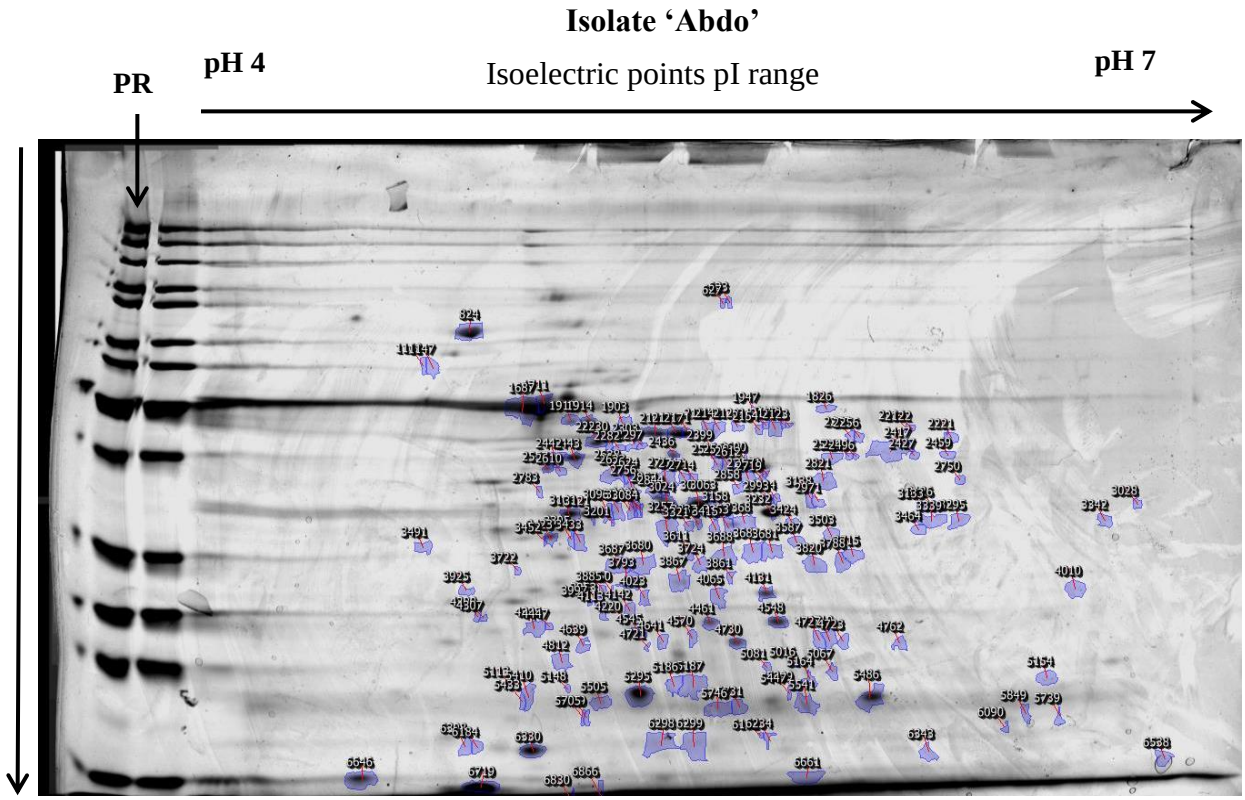
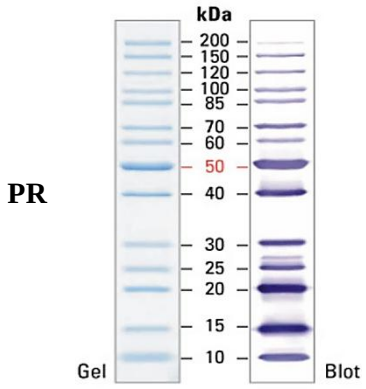


Figure 5.2: Silver stained 2-D gel showing intracellular proteins from UK isolate 'Abdo'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

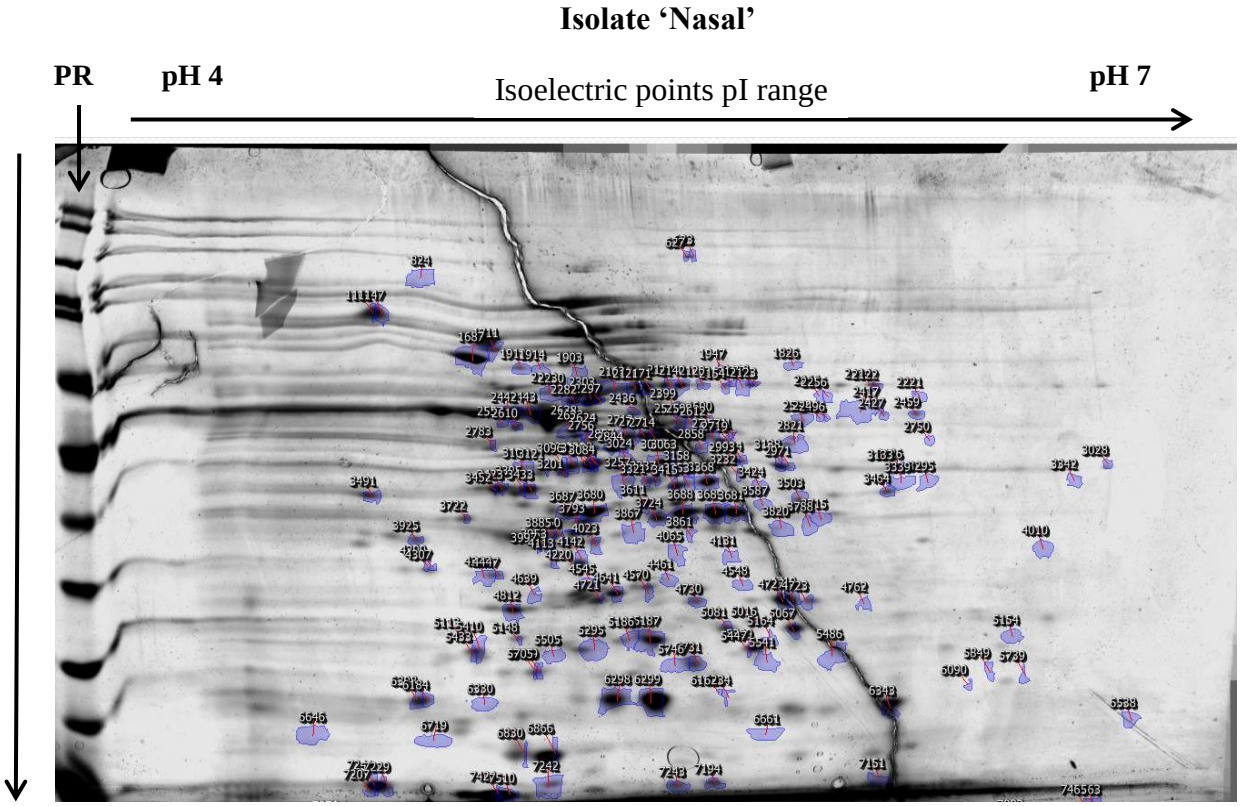
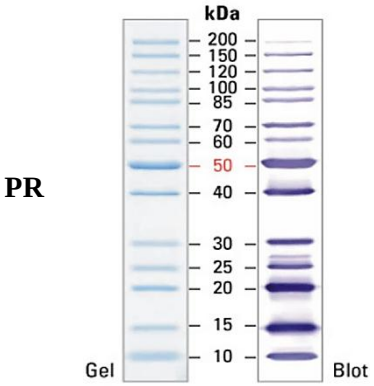


Figure 5.3: Silver stained 2-D gel showing intracellular proteins from UK isolate 'Nasal'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

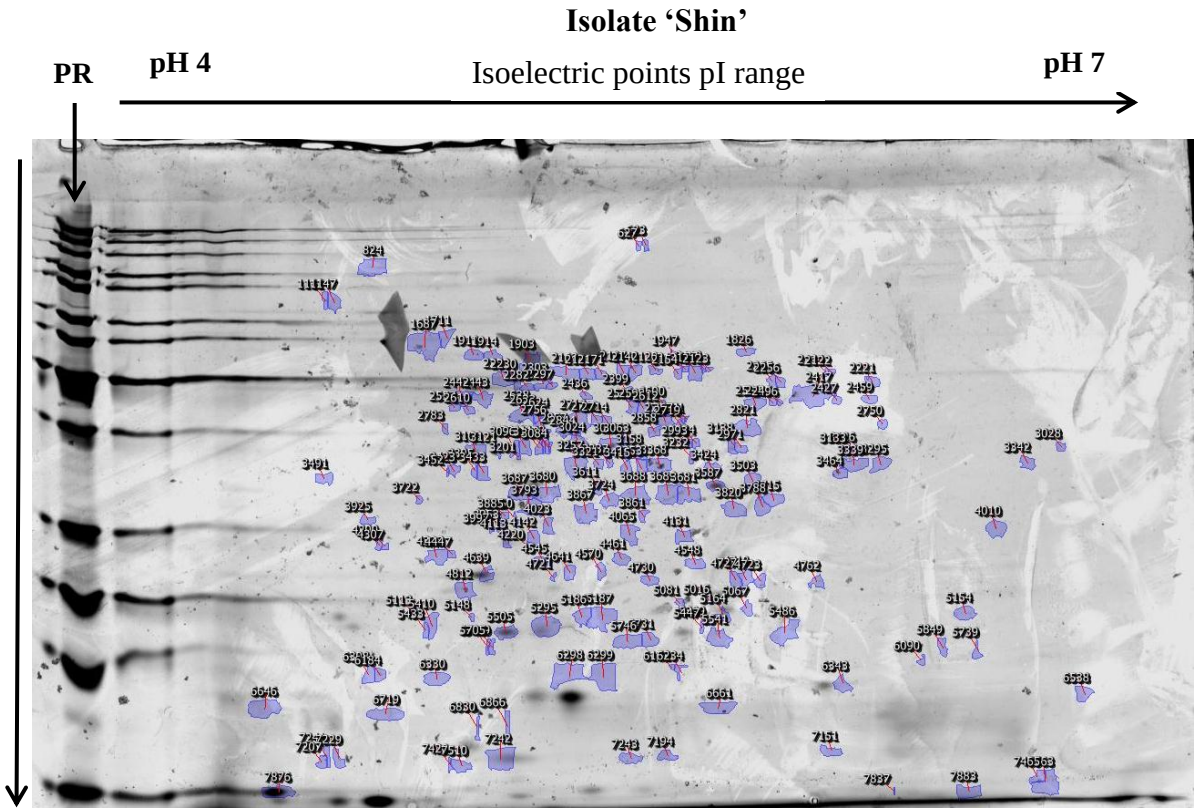
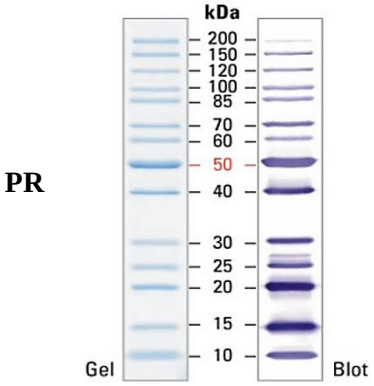


Figure 5.4: Silver stained 2-D gel showing intracellular proteins from UK isolate 'Shin'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points pI range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

Table 5.1: Protein spots selected from the 7904 spots detected by the Progenesis SameSpot software for the UK MRSA group (consisting of isolates 16/11, Abdo, Nasal, Shin)

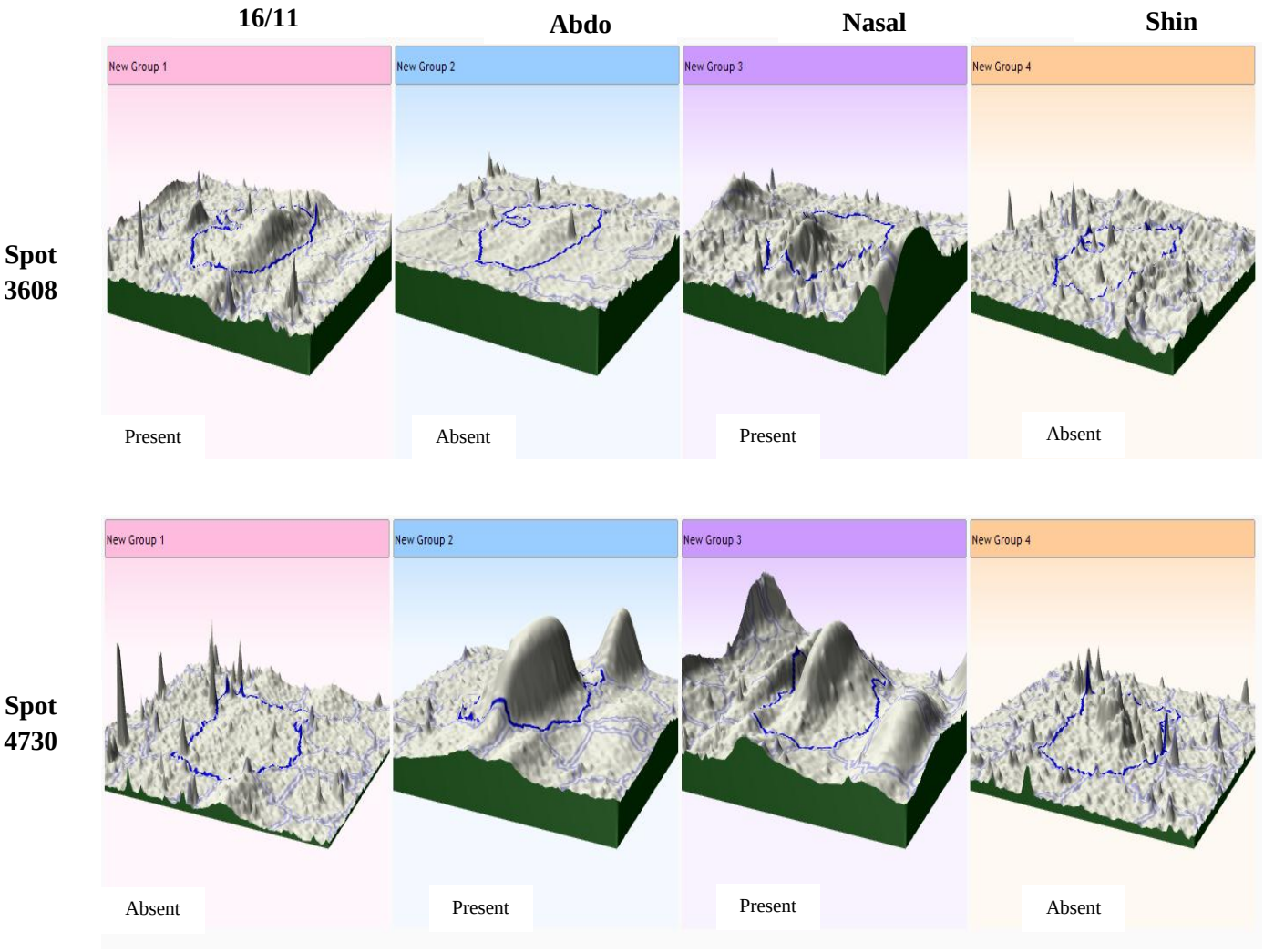
	UK MRSA isolates				
	Spot	**16/11**	**Abdo**	**Nasal**	**Shin**
	623	-	-	+	-
	824	-	+	-	-
	1162	-	-	+	-
	1187	-	-	+	-
	1826	+	-	-	-
	1912	-	+	+	-
	2140	-	-	+	-
	2171	-	+	+	-
	2253	-	+	-	-
	2255	+	-	-	-
	2443	+	+	-	-
	2459	+	-	+	-
	2503	+	-	+	-
	2599	+	+	-	-
	2624	-	+	+	-
	2712	+	+	+	-
	2821	+	-	+	-
	2857	-	-	+	-
	3024	-	+	+	-
	3085	-	+	+	-
+: Present	**3109**	-	+	-	-
	3138	-	-	+	-
-: Absent	**3158**	-	+	-	-
	3237	-	-	+	-
	3295	+	-	-	-
	3339	+	-	-	-
	3509	+	-	+	-
	3587	-	-	-	+
	3608	+	-	+	-
	3788	+	-	-	-
	4113	-	-	+	+
	4461	-	+	-	-
	4545	-	+	-	-
	4639	-	-	-	+
	4730	-	+	+	-
	5154	+	-	-	-
	5186	+	-	+	-
	5295	-	+	+	+
	5486	-	+	-	-
	5505	-	-	-	+
	6299	-	-	+	-
	6330	-	+	-	-
	7151	+	-	-	-
	7194	-	-	+	-
	7207	-	-	+	-
	7242	-	-	+	+
	7420	-	-	+	-
	7465	+	-	-	-
	7876	-	-	-	+
	7883	+	-	-	-

Table 5.2: Selected protein spots and their corresponding Isoelectric point (pI) and molecular weight (mw); UK group

UK MRSA isolates		
Spot	**pI**	**Mw (kDa)**
623	5.6	150
824	5.1	85
1162	4.7	85
1187	4.7	72
1826	5.9	60
1912	5.5	53
2140	5.6	56
2171	5.5	60
2253	6.25	45
2255	5.9	50
2443	5.1	50
2459	6.25	48
2503	5.1	50
2599	5.9	50
2624	5.3	50
2712	5.7	45
2821	5.9	48
2857	5.2	45
3024	5.4	37
3085	5.5	38
3109	5.4	37
3138	5.9	45
3158	6.8	37
3237	5.5	39
3295	5.4	40
3339	6.2	40
3509	6	39
3587	5.9	35
3608	5.4	40
3788	5.6	39
4113	5.3	32
4461	5.7	25
4545	5.5	25
4639	5.1	24
4730	5.6	24
5154	6.5	25
5186	5.5	25
5295	5.4	23
5486	6.25	20
5505	5.2	22
6299	5.6	19
6330	5	20
7151	6.1	17
7194	5.3	15
7207	4.6	15
7242	5.2	15
7420	5.1	15
7465	6.2	15
7876	4.4	15
7883	6.5	15

Figure 5.5: 3D representations of protein spots detected by the Progenesis SameSpot software

UK group



5.3.2. Nigerian group

When it came to the Nigerian group, isolate 114 was seen to have only 14 spots of the 50 selected spots, isolates 193, S54N and GM12 were seen to have 19, 22 and 24 spots respectively (Table 5.3). While isolates GM12 and S54N had spots 1231, 1434, 1532, 1547, 1611, 1631, 1783, 1894, 1916, 1932, 1938, 2485 and 2575 in common, spots 1424, 1434, 1450, 2123 and 2723 were common to isolates 114 and S54N (Table 5.3). Isolates GM12 and 193 had spots 1532, 1611, 1715, 1932, 2539 and 3092 in common. While only spots 1385, 1403 and 2389 were common to isolates 114 and 193, and spots 1434, 2162 and 2291 were common to isolates GM12 and 114 (Table 5.3). In addition, spots 1532 and 1611 were common to 193, GM12 and S54N, while only spot 1434 was common to isolates 114, GM12 and S54N (Table 5.3). The spot with the highest molecular weight (i.e. spot 1232; 51 kDa [Table 5.4)]) among the 50 spots selected for the Nigerian group was seen to be present in both isolates GM12 and S54N (Table 5.3); the spots with the lowest molecular weights (i.e. spots 3203 and 3214; 16 kDa [Table 5.4]) were also found to be present in only isolates GM12 (spot 3214) and isolate S54N (spot 3203) as well (Table 5.3).

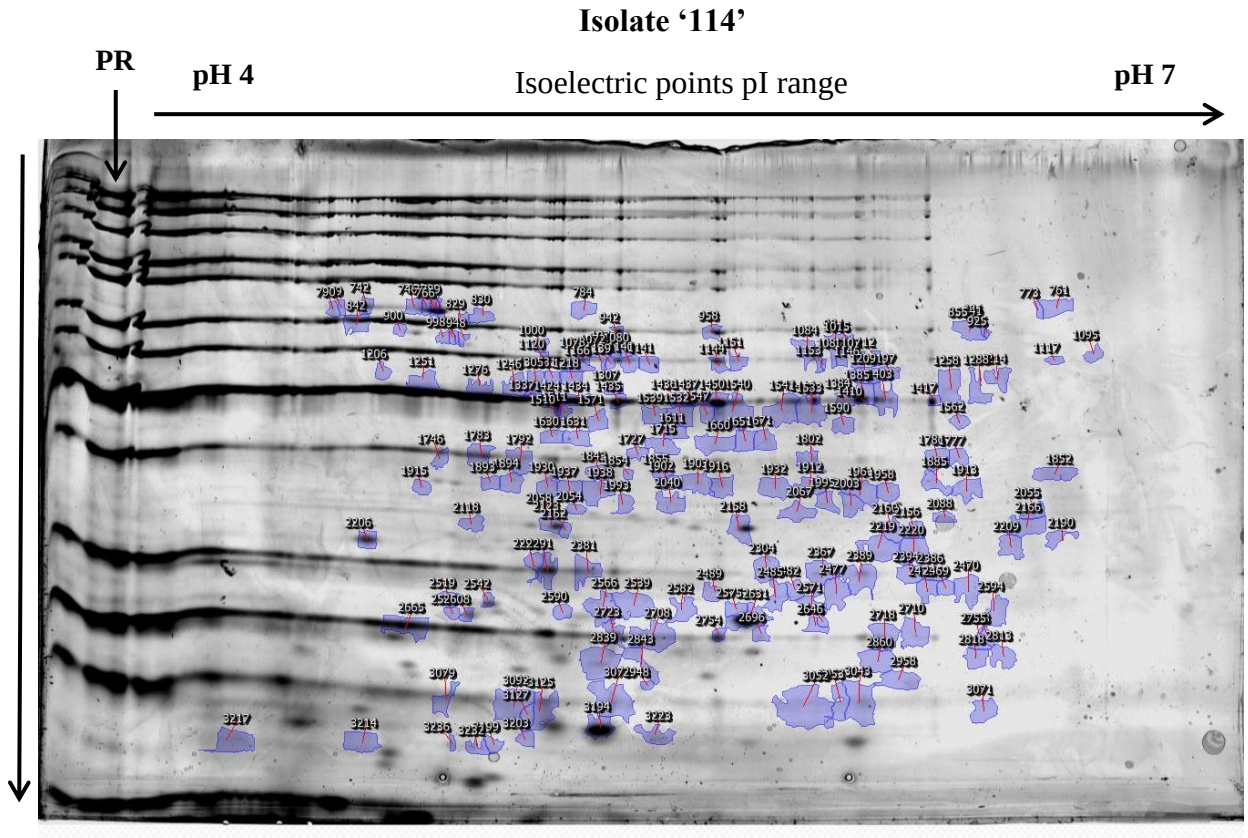
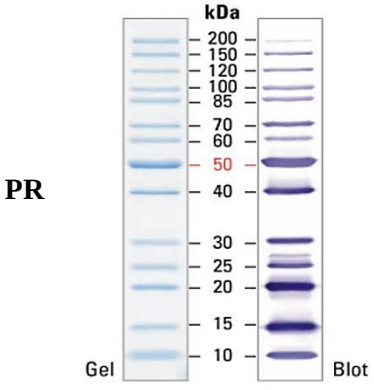


Figure 5.6: Silver stained 2-D gel showing intracellular proteins from Nigerian isolate '114'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

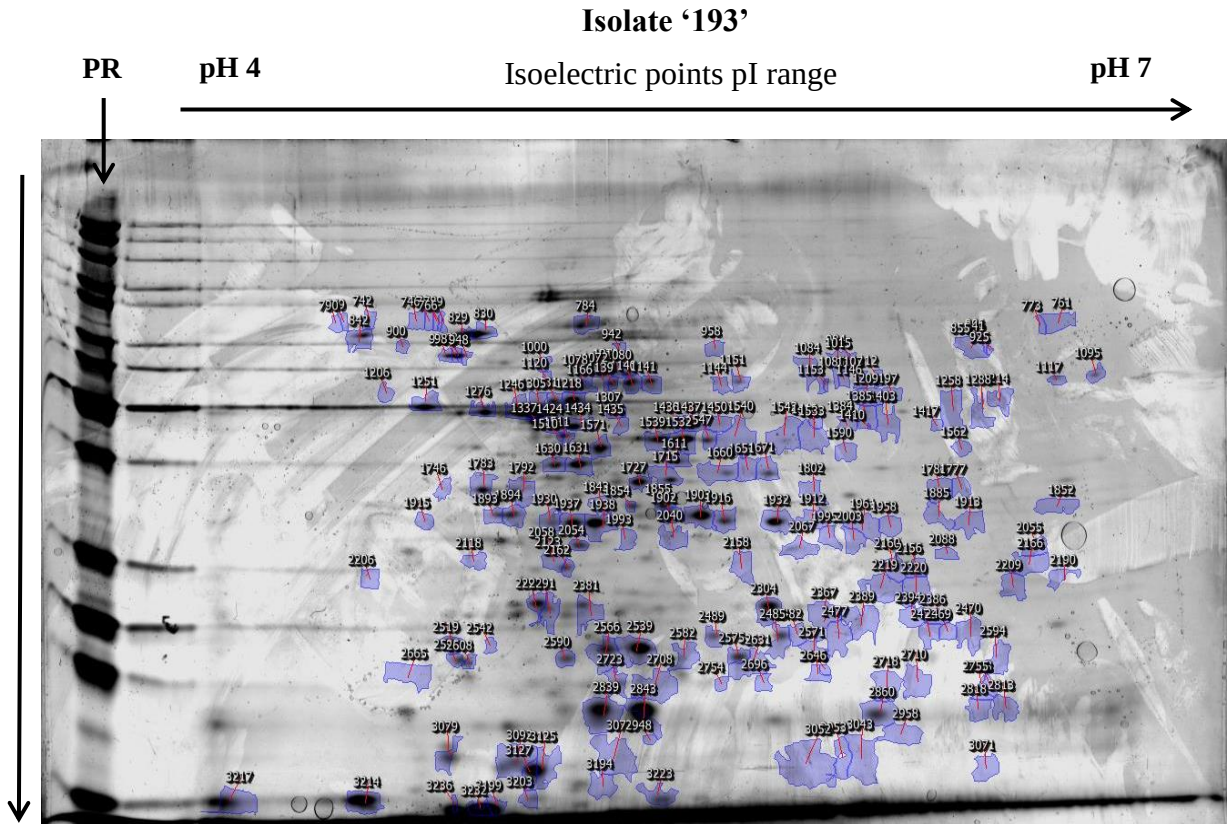
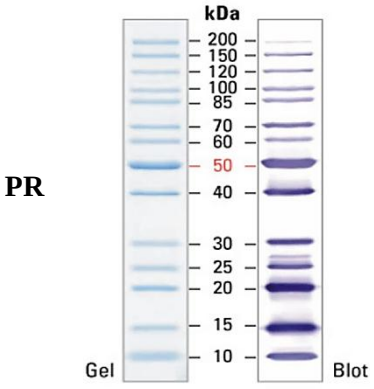


Figure 5.7: Silver stained 2-D gel showing intracellular proteins from Nigerian isolate '193'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

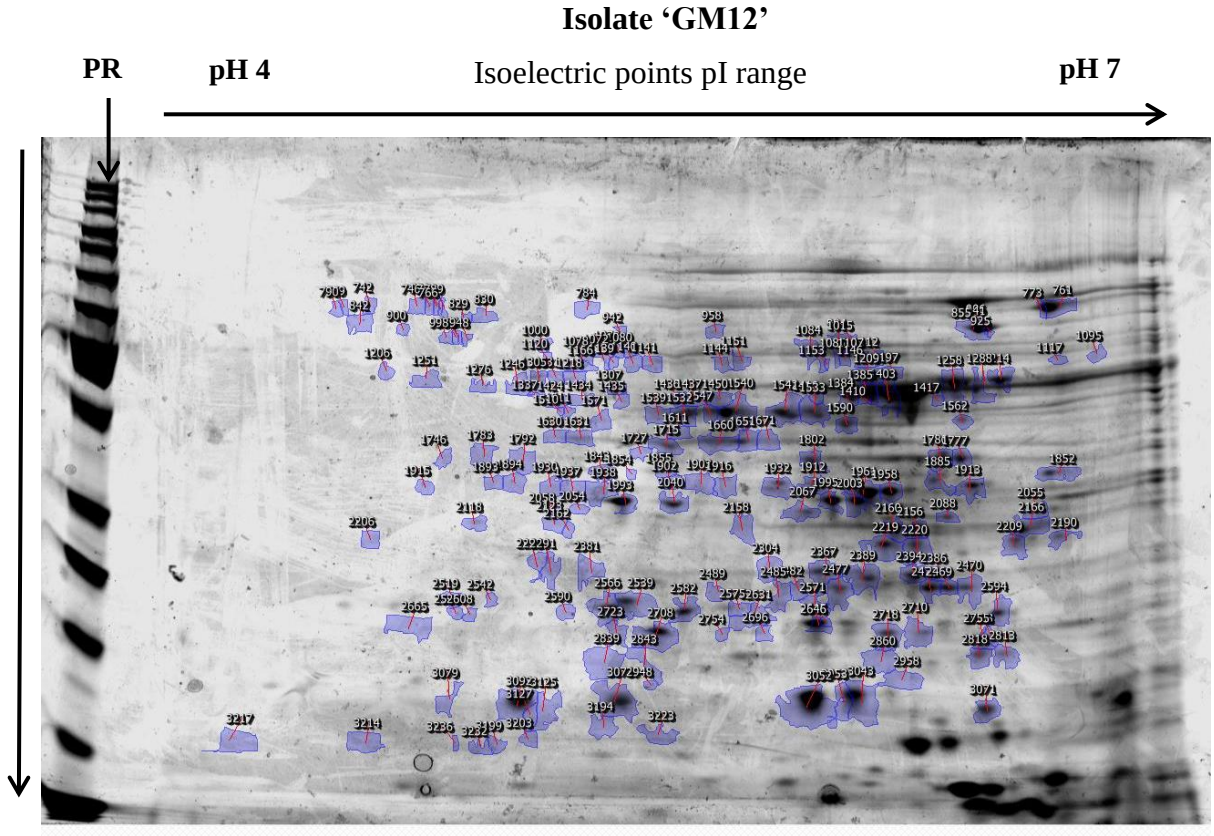
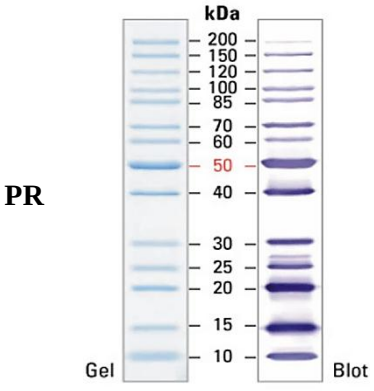


Figure 5.8: Silver stained 2-D gel showing intracellular proteins from Nigerian isolate 'GM12'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

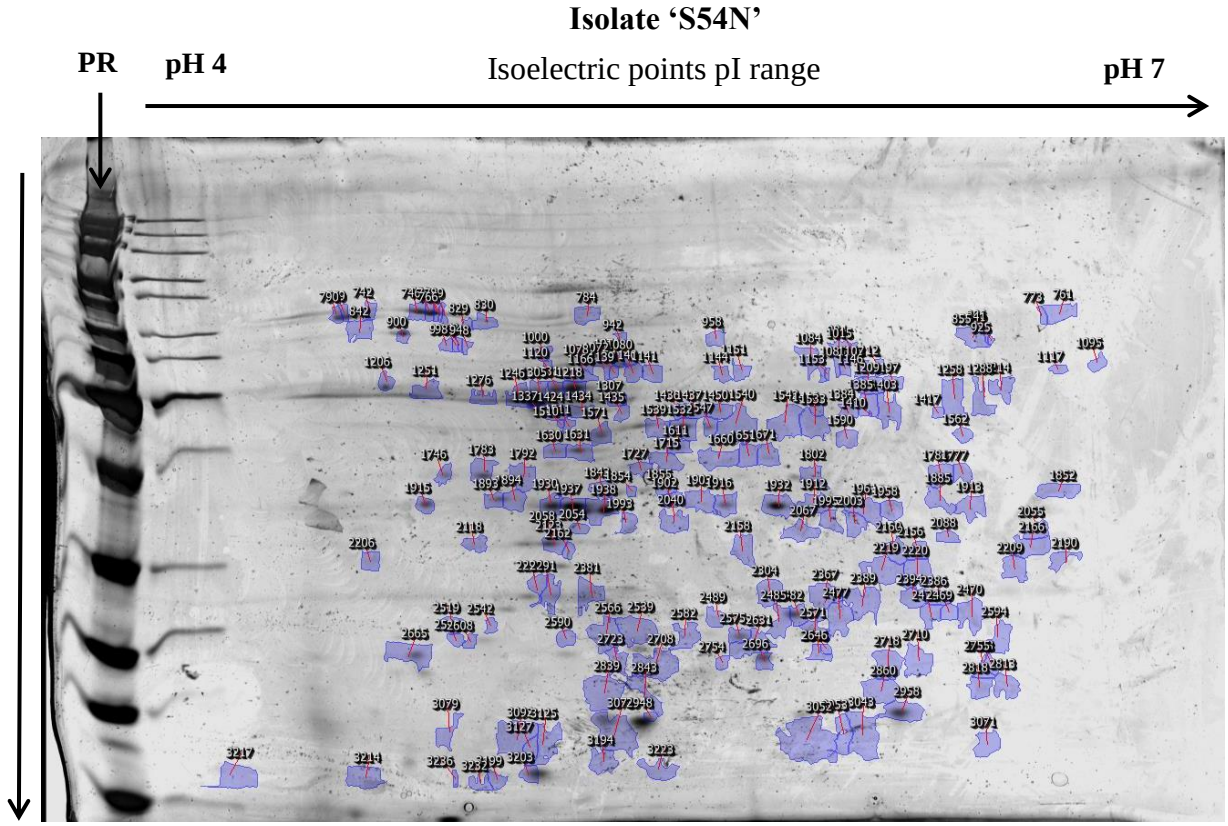
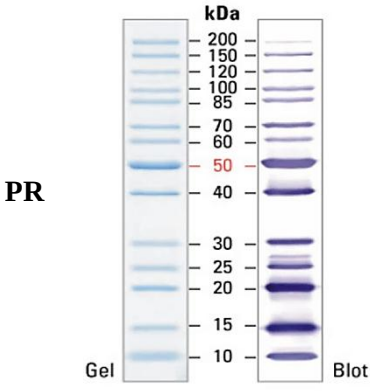


Figure 5.9: Silver stained 2-D gel showing intracellular proteins from Nigerian isolate 'S54N'; protein spots are circled in blue. Proteins were separated in the first dimension on a nonlinear pH 4–7 immobilized pH gradient (IPG) strip and the Progenesis SameSpot software was used in spot analysis. Isoelectric points (pI) range is shown on the top, while the direction of second-dimension SDS-PAGE is represented by an arrow on the left.



PR: PageRuler Unstained Protein Ladder (Thermo Fisher Scientific)

Table 5.3: Protein spots selected from the 3251 spots detected by the Progenesis SameSpot software for the Nigerian MRSA group (consisting of isolates 114, 193, GM12, S54N)

Nigerian MRSA isolates				
Spot	**114**	**193**	**GM12**	**S54N**
1231	-	-	+	+
1276	-	-	+	-
1385	+	+	-	-
1403	+	+	-	-
1424	+	-	-	+
1434	+	-	+	+
1437	-	-	+	-
1450	+	-	-	+
1532	-	+	+	+
1533	-	+	-	-
1542	-	+	-	-
1547	-	-	+	+
1611	-	+	+	+
1631	-	-	+	+
1715	-	+	+	-
1727	-	-	+	-
1777	-	+	-	-
1783	-	-	+	+
1786	-	+	-	-
1843	-	+	-	-
1852	-	+	-	-
1894	-	-	+	+
1915	-	-	-	+
1916	-	-	+	+
1930	-	-	-	+
1932	-	+	+	+
1938	-	-	+	+
1961	-	+	-	-
2123	+	-	-	+
2158	+	-	-	-
2162	+	-	+	-
2166	-	+	-	-
2206	+	-	-	-
2219	-	+	-	-
2290	-	-	+	-
2291	+	-	+	-
2304	-	-	-	+
2389	+	+	-	-
2485	-	-	+	+
2539	-	+	+	-
2542	+	-	-	-
2575	-	-	+	+
2723	+	-	-	+
2958	-	-	-	+
3052	-	+	-	-
3092	-	+	+	-
3127	-	-	+	-
3194	+	-	-	-
3203	-	-	-	+
3214	-	-	+	-

+: Present

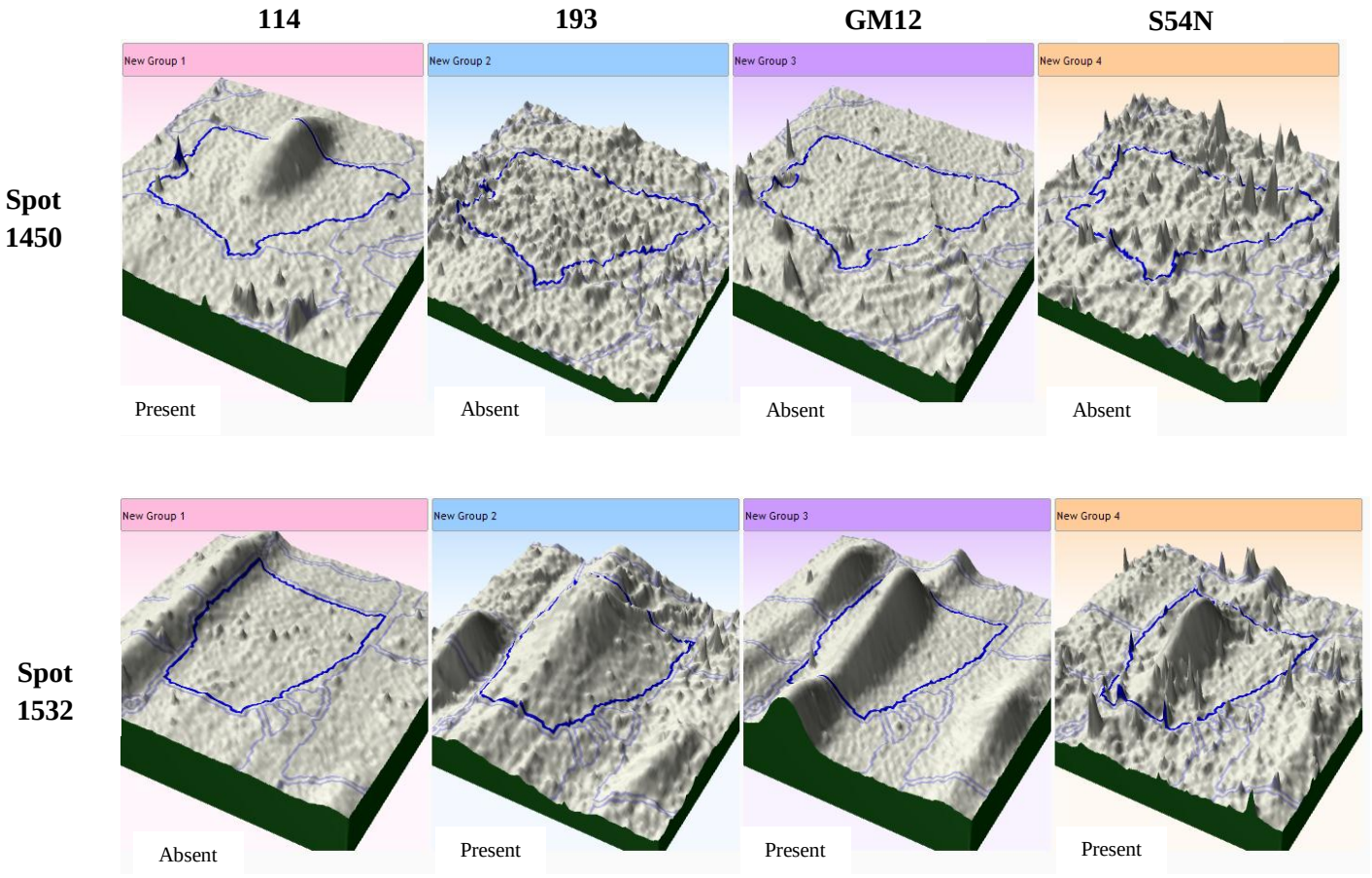
-: Absent

Table 5.4: Selected protein spots and their corresponding Isoelectric point (pI) and molecular weight (mw); Nigerian group

Nigerian MRSA isolates		
Spot	**pI (pH)**	**mw (kDa)**
1231	4.8	51
1276	5.1	50
1385	6.3	53
1403	6.1	50
1424	5.3	52
1434	5.4	52
1437	5.6	50
1450	5.8	48
1532	5.6	48
1533	6	50
1542	5.9	50
1547	5.3	48
1611	5.6	45
1631	5.4	40
1715	5.6	39
1727	5.5	39
1777	6.4	39
1783	5.1	39
1786	6.4	39
1843	5.4	38
1852	6.6	38
1894	5.3	35
1915	4.9	36
1916	5.8	35
1930	5.3	35
1932	6	35
1938	5.4	35
1961	6.1	36
2123	5.3	33
2158	5.7	30
2162	5.3	30
2166	6.6	30
2206	4.8	28
2219	6.2	28
2290	6.7	29
2291	5.3	27
2304	5.9	27
2389	6.3	27
2485	6	27
2539	5.5	24
2542	5.1	24
2575	5.8	23
2723	5.4	24
2958	6.4	20
3052	6.1	18
3092	5.2	18
3127	5.2	17
3194	5.4	17
3203	5.2	16
3214	4.6	16

Figure 5.10: 3D representations of protein spots detected by the Progenesis SameSpot software.

Nigerian group



5.3.3. Comparison of the protein spots selected for the UK and Nigerian groups

A comparison of the characteristics of the protein spots selected for the UK and Nigerian groups showed that the UK spots 2255 and 2599 had identical pI and mw with Nigerian spot 1542 i.e. pI = 5.9 and mw = 50 kDa (Table 5.5). This was also the case with UK spot 2443 and Nigerian spot 1276 which both had identical pI of 5.1 and mw 50 kDa; UK spot 4639 and Nigerian spot 2542 both also had identical pI of 5.1 and mw 24 kDa (Table 5.5). In some instances, although the pIs and mws were not identical, they were very closely similar. For instance UK spot 3587 (pI = 5.9, mw = 35 kDa) and Nigerian spot 1916 (pI = 5.8, mw = 35 kDa), UK spot 7151 (pI = 5.1, mw = 17 kDa) and Nigerian spot 3052 (pI = 5.1, mw = 18 kDa) as well as UK spot 7242 (pI = 5.2, mw = 15 kDa) and Nigerian spot 3203 (pI = 5.2, mw = 16 kDa) (Table 5.5).

Table 5.5: Side by side presentation of protein spots selected for the UK and Nigerian MRSA groups along with their corresponding Isoelectric point (pI) and molecular weight (mw). Protein spots with identical pIs and mws are highlighted in identical colours while protein spots with nearly identical pIs and mws have the same text colour.

UK group			Nigerian group		
Spot	**pI**	**Mw (kDa)**	**Spot**	**pI**	**mw (kDa)**
623	5.6	150	**1231**	4.8	51
824	5.1	85	**1276**	5.1	50
1162	4.7	85	**1385**	6.3	53
1187	4.7	72	**1403**	6.1	50
1826	5.9	60	**1424**	5.3	52
1912	5.5	53	**1434**	5.4	52
2140	5.6	56	**1437**	5.6	50
2171	5.5	60	**1450**	5.8	48
2253	6.25	45	**1532**	5.6	48
2255	5.9	50	**1533**	6	50
2443	5.1	50	**1542**	5.9	50
2459	6.25	48	**1547**	5.3	48
2503	5.1	50	**1611**	5.6	45
2599	5.9	50	**1631**	5.4	40
2624	5.3	50	**1715**	5.6	39
2712	5.7	45	**1727**	5.5	39
2821	5.9	48	**1777**	6.4	39
2857	5.2	45	**1783**	5.1	39
3024	5.4	37	**1786**	6.4	39
3085	5.5	38	**1843**	5.4	38
3109	5.4	37	**1852**	6.6	38
3138	5.9	45	**1894**	5.3	35
3158	6.8	37	**1915**	4.9	36
3237	5.5	39	**1916**	**5.8**	**35**
3295	5.4	40	**1930**	5.3	35
3339	6.2	40	**1932**	6	35
3509	6	39	**1938**	5.4	35
3587	**5.9**	**35**	**1961**	6.1	36
3608	5.4	40	**2123**	5.3	33
3788	5.6	39	**2158**	5.7	30
4113	5.3	32	**2162**	5.3	30
4461	5.7	25	**2166**	6.6	30
4545	5.5	25	**2206**	4.8	28
4639	5.1	24	**2219**	6.2	28
4730	5.6	24	**2290**	6.7	29
5154	6.5	25	**2291**	5.3	27
5186	5.5	25	**2304**	5.9	27
5295	**5.4**	**23**	**2389**	6.3	27
5486	6.25	20	**2485**	6	27
5505	5.2	22	**2539**	5.5	24
6299	5.6	19	**2542**	5.1	24
6330	5	20	**2575**	5.8	23
7151	**6.1**	**17**	**2723**	**5.4**	**24**
7194	5.3	15	**2958**	6.4	20
7207	4.6	15	**3052**	**6.1**	**18**
7242	**5.2**	**15**	**3092**	5.2	18
7420	5.1	15	**3127**	5.2	17
7465	6.2	15	**3194**	5.4	17
7876	4.4	15	**3203**	**5.2**	**16**
7883	6.5	15	**3214**	4.6	16

Two-dimensional electrophoresis (2-DE) is a powerful and widely used method for analysis of complex protein mixtures with exceptional ability to separate thousands of proteins at once (Magdeldin *et al*., 2014). The 2-DE technique is unique in its ability to detect post- and co-translational protein modifications, which cannot be predicted from the genome sequence (Magdeldin *et al*., 2014; Kumar *et al*., 2017).

It is known that majority of *S. aureus* proteins are clustered between pH 4 and 6 (Assafi, 2016). Therefore, as expected, results from this study showed that with the exception of protein spots 3158, 5154, 7883 (UK group), 1852, 2166 and 2290 (Nigerian group) which had pI values $\geq$ 6.5, all the other selected protein spots had pI values within the 4 to 6.4 range (Table 5.5). Furthermore, Rosen *et al*., 2004 have previously shown that *S. aureus* proteins that clustered between pH 4 and 6 includes many cellular proteins, such as chaperones, biosynthetic, and metabolic enzymes. The ExPASy Tagldent tool (https://web.expasy.org/tagident/) was used to predict the identity of protein spots 2255, 2599 (UK group) and 1542 (Nigerian group) which had identical pI (5.9) and mw (50 kDa). One of the predicted proteins, UDP-N-acetylmuramate--L-alanine ligase (pI = 5.94; mw = 49617 Da) is an essential, cytoplasmic peptidoglycan biosynthetic enzyme, that catalyses the adenosine triphosphate (ATP)-dependent ligation of L-alanine (Ala) and UDP-N-acetylmuramic acid (UNAM) to form UDP-N-acetylmuramyl-L-alanine (UNAM-Ala). Additionally, one of the proteins predicted by the ExPASy Tangldent tool for UK spot 2443 and Nigerian spot 1276 (identical pI of 5.1 and mw 50 kDa) is the enzyme ATP synthase subunit beta 1 (pI = 5.05; mw = 50170 Da), which is found in the plasma membrane of bacteria, catalyses the synthesis of ATP from adenosine diphosphate (ADP) using the proton-motive force generated by substrate-driven electron transfer chains. Majority, if not all, the proteins predicted using the ExPASy Tagldent tool for all the protein spots selected in this study were enzymes that are in one way or the other involved in metabolic pathways.

Hecker *et al*., 2010 and Atshan *et al*., 2015 have previously demonstrated that different clinical isolates of *S. aureus* within the same clonal complex have extremely different exoproteome profiles. Furthermore, results from the study by Ziebandt *et al*., 2010 showed that a high degree of heterogeneity exists in the exoproteome of clonally different *S. aureus* strains. Although the intracellular rather than the extracellular protein profiles of the MRSA isolates involved in this study were assessed, the results obtained showed that isolates belonging to different clonal complexes had different intracellular protein profiles. For instance, as presented in Table 5.1, UK isolate Nasal (a nasal isolate belonging to the CC22 lineage) had only three proteins (spot numbers 4113, 5295 and 7242) in common with isolate Shin (a wound isolate belonging to the CC30 lineage). In addition, 27 of the 50 selected spots were detected in isolate Nasal compared to only six of the 50 selected spots that were detected in isolate Shin (Table 5.1). Furthermore, the untyped Nigerian isolate S54N (a wound isolate), was seen to have only three proteins (spot numbers 1532, 1611 and 1932) in common with isolate 193, a high vaginal swab isolate which belongs to the ST239 lineage (Table 5.3). Similarly, the untyped wound isolate GM12 had only three proteins (spot numbers 1434, 2162 and 2291) in common with isolate 114, a wound isolate of the ST239 lineage (Table 5.3). Moreover, 24 of the 50 selected protein spots were present in isolate GM12 compared to the 14 that were detected in isolate 114 (Table 5.3). Variability was also observed in the protein profiles of isolates of the same lineage, i.e. Nigerian isolates 114 and 193, that both belong to the ST239 lineage had only three proteins (spot numbers 1385, 1403 and 2389) in common (Table 5.3). As put forward by Atshan *et al*., 2015, this variability observed in the intracellular protein profiles of isolates from different lineages in terms of the number, presence and absence of spots, suggests that the gene control systems may not be similar among the types of *S. aureus* clones and this could strongly disturb protein production and/or expression.

The UK isolate Nasal (the CC22 lineage) was seen to have seven and eight protein spots in common with untyped isolates 16/11 and Abdo respectively (Table 5.1). This points to the

possibility that the untyped isolates may have similar gene control systems as isolate Nasal. Additionally, because they have several protein spots in common and regardless of the fact that variation exists in the protein profiles of isolates within the same lineage (as exemplified by isolates 114 and 193), the possibility that isolates 16/11 and Abdo may belong to a lineage closely related to CC22 cannot be ruled out. Untyped Nigerian isolates GM12 and S54N had 13 protein spots in common (Table 5.3). This finding also points to the possibility that isolates GM12 and S54N may belong to the closely related, if not the same lineage and that they may have similar gene control systems.

Nigerian isolates GM12 and S54N, both of which were isolated from wounds (Table 2.1) shared more proteins (13) in common than any two other isolates in this study (Table 5.3). A look at their virulence factor profiles showed that both isolates were identical except for the presence of the enterotoxin B gene borne by isolate S54N (Chapter 2; Table 2.5). Despite these close similarities, they showed distinct patterns of toxicity on mammalian cells as seen in the cytotoxicity experiments described in chapter four. Six and 24 hrs post-infection at both MOI 10:1 and 100:1, isolate GM12 (live) was more toxic than isolate S54N (live) on KB cells (Tables 4.1 – 4.4). The reverse was the case when it came to A549 cells as it was seen that isolate S54N (live) was more toxic than isolate GM12 (live) (Tables 4.6 – 4.9). Therefore, the fact that these isolates have a good number of proteins and virulence factors in common did not translate to close patterns on toxicity on mammalian cells. Rather, they showed distinct cell tropism.

The position of a spot in the 2D map is not the enough information for an exact identification of a protein (Lopez, 2007). However, because 2-DE gel easily and efficiently couples with many other analysis and biochemical techniques, it provides a compatible platform for subsequent analysis (Magdeldin *et al*., 2014). Therefore, association of 2-DE technique with techniques such as mass spectrometry, in combination with computer-assisted software for image evaluation, has enabled 2-DE analysis in comprehensive qualitative and quantitative examination of proteomes as well as

in separation and selection of proteins (Kumar *et al*., 2017). Unfortunately, financial constraints prevented further analysis of protein spots in this study.

Although it is a widely used technique, the 2-DE technique is associated with poor solubility of membrane proteins, limited dynamic range and difficulties in displaying and identifying low-abundance proteins (Lopez, 2007; Magdeldin *et al*., 2014) as well as gel-to-gel variability (Mesri *et al*., 2014; Magdeldin *et al*., 2014). Nevertheless, recent advances have led to the development of modified 2-DE techniques, such as the differential imaging gel electrophoresis (DIGE) technique, Non-equilibrium pH gel electrophoresis (NEPHGE) technique and Blue-Native polyacrylamide gel electrophoresis, to overcome some of the limitations associated with 2-DE (Magdeldin *et al*., 2014).

In conclusion, findings from this study showed that variation exists in the intracellular protein profiles of MRSA isolates from the same and different lineages

Chapter Six

Prospective MRSA Treatment Option

6.1. Introduction

Although several therapeutic strategies exist for the treatment of MRSA infections, the use of antibiotics is effective and the most historically important (Dou *et al*., 2015). However, in addition to being resistant to virtually all β-lactam antibiotics, MRSA can and has acquired resistance to multiple alternative antibiotics (tetracyclines, macrolides, streptogramins, lincosamides, aminoglycosides), thereby complicating treatment of infection. A further complication of the MRSA antibiotic therapy is the unsettling trend of decreased antibiotic research and development (Morell and Balkin, 2010; Dou *et al*., 2015). In view of the current state of MRSA therapy, there is a need to develop novel anti-MRSA strategies, and one of such is the iron-chelation therapy.

With only a few exceptions, iron, the most common redox active metal found in proteins (Frawley and Fang, 2014), is now absolutely required by life of all forms (Andrews *et al*., 2003). It acts as a co-factor within the active site of key enzymes involved in critical biochemical pathways of DNA synthesis, oxygen transport and energy generation (Kalinowski and Richardson, 2005). Nevertheless, iron can be toxic to cells at high concentrations as it participates in Fenton reactions that generate oxygen radicals which causes macromolecular damage and cell death (Lin *et al*., 2011; Vasileva *et al*., 2012; Pi and Helmann, 2017). In addition to being a critical nutrient source for micro-organisms, iron also enhances microbial virulence and impairs host antimicrobial responses (Luo *et al*., 2014). Data from several studies indicate that iron acquisition is essential for multiple stages of *S. aureus* virulence and pathogenicity (Luo *et al*., 2014). Consequently, limiting the amount of available iron ought to, in principle, inhibit microbial growth (Qiu *et al*., 2010). The concentration of iron freely accessible in the mammalian host is much lower than the optimal iron concentration for growth of most bacteria (Parrow *et al*., 2013). Therefore, in order to grow under the iron-restricted conditions of the human body, most bacteria have developed specialised high-affinity iron-uptake systems (Kim and Shin, 2009). Examples of iron-uptake systems possessed by

S. aureus include the haeme or haemoprotein-specific staphylococcal receptor, the iron-regulated surface determinant system and the transferrin receptor (Luo *et al.,* 2014). These bacterial iron-uptake systems are potential targets for the development of preventive or therapeutic agents especially for multi-drug resistant bacteria (Kim and Shin, 2009; Cassat and Skaar, 2013) such as MRSA. In the same vein, iron-chelation therapies that are able to prevent iron-uptake by bacteria have received and are still receiving considerable attention as potential novel preventive and therapeutic measures. (Etz *et al*., 2002; Marx, 2002; Chan *et al*., 2009; Qiu *et al*., 2010).

The design of iron chelating polymers has previously been based on strategies, such as the immobilization of natural chelators onto activated supports (Horowitz *et al*., 1985; Hallaway *et al*., 1989; Li *et al*., 2016), conjugation of bidentate ligands with activated polymers (Winston and Kirchner, 1978; Feng *et al*., 1994; Li *et al*., 2016) and copolymerization of 1-(-acrylamidoethyl)-3-hydroxy-2-methyl-4(1*H*)-pyridinone with other cross-linking agents (Polomoscanik *et al*., 2005; Li *et al*., 2016). The structures generated from the immobilization of natural chelators onto activated supports are limited to carbohydrate matrices, consequently, they are expensive to prepare, and high binding capacities are difficult to achieve (Li *et al*., 2016). Furthermore, difficulty associated with the control of the chemistry for the second and third approaches leads to the generation of polydisperse polymers as well as crosslinked materials with poor solubility. Thus, the need to develop a simple preparative method for iron-binding polymers of well-defined structure, controlled molecular weights and low poly-dispersities (Li *et al*., 2016).

Poly(glycidyl methacrylate) (PGMA) is a well-known polymer for both industrial and biomedical applications because it is reactive, inexpensive, hydrophilic, biocompatible, and generally nontoxic; it also permits fast and efficient post-polymerization modifications (Gao *et al.*, 2011; Benaglia *et al*., 2013). On the other hand, 3-Hydroxypyridin-4-ones (HPOs) are one of the main classes of candidates for the development of orally active iron chelators, and 1,2-dimethyl-

3-hydroxypyridin-4-one (Deferiprone) is currently available for clinical use (Apotex Inc., Toronto, Canada as Ferriprox) (Zhou *et al*., 2012).

Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerisation has previously been successfully employed for the synthesis of well-defined PGMA polymers, exerting remarkable control over their molecular weights and affording polymers with low molecular-weight distributions. (Zhu *et al*., 2004; Yin *et al*., 2007). Furthermore, both linear- and star-shaped PGMAs have previously been modified with different amines by nucleophilic ring-opening reaction of the epoxy group (An *et al*., 2011; Li *et al*., 2013). By combining these strategies, each novel macromolecular iron-chelator used in this study was synthesised by conjugating an amine-substituted 3-Hydroxypyridin-4-one (HPO: type 1, 5 or 5b) to a Poly (glycidyl methacrylate) (PGMA: type 7 or 8) in a ring-opening reaction using the RAFT polymerisation technique (Li *et al*., 2013). Therefore, each polymer combines both an amine and PGMA functionality.

The aim of this study was to assess the anti-MRSA potential of five novel macromolecular iron-chelators synthesised using the RAFT polymerisation technique. The cytotoxic effects of theses chelators on a mammalian cell line was also assessed.

6.2. Materials and Methods

All chemicals were purchased from Sigma, unless otherwise stated.

6.2.1. Cell lines and cell culture

Human KB epithelial cell line (carcinoma cells) were used in this study and were cultured, maintained, passaged and seeded in 24-well plates as described in Chapter Three (sections 3.2.1, 3.2.2, 3.2.3).

6.2.2. Bacterial strains and culture

In consideration of the fact that this study aimed to ascertain the anti-MRSA potential of novel iron-chelators and the fact that only small quantities of the novel iron-chelators were available, only one MRSA isolate (UK isolate Nasal) was used in this study. The isolate was grown and maintained as described in section 2.2.1 (Chapter Two). Optical densities of bacterial broth cultures were measured in a spectrophotometer as previously described in the same section i.e. OD_{595} 0.22 = ~10^8 CFU/ml.

6.2.3. Iron-chelators (iChs)

The antimicrobial effect of five novel iChs [71 (molecular weight (mw) = 50,100 Da), 75 (mw = 33,100 Da), 85 (mw = 8,900 Da), 70 (mw = 34,600 Da) and 81 (mw = 13,900 Da)], provided by Professor Robert Hider, King's College, London, were assessed in the course of this study. All five previously untested iChs were synthesised by conjugating an amine-substituted 3-Hydroxypyridin-4-one (HPO: type 1, 5 or 5b) to a Poly (glycidyl methacrylate) (PGMA: type 7 or 8) in a ring-opening reaction using the Reversible Addition-Fragmentation Chain Transfer (RAFT) polymerisation technique (Li *et al*., 2013). The 3-hydroxyl group on the HPO ring of HPO type 5 was methylated to form HPO type 5b which is present in iCh 70. The pFe^{3+} (i.e. the negative logarithm of concentration of free iron (III) in solution under defined conditions) value of HPO type 1 was found to be 19.47 while that of HPO type 5 was found to be 15.9. The iCh deferiprone

(3-Hydroxy-1,2-dimethyl-4(1H)-pyridone; mw=139.152 Da), an HPO with pFe^{3+} 20.5 (Hider, 2014), clinically available for treatment of iron overload in humans, was used as a control in this study.

6.2.4. Preparation of iron-chelators for determination of antimicrobial activity

Stock solutions of the iChs were prepared by dissolving in appropriate solvents i.e. 20% dimethyl sulfoxide (DMSO) in water (deionised) for 71 and 85, 50% DMSO in water (deionised) for both 75 and 70, and water (deionised) for both 81 and Deferiprone. The amounts of DMSO present in the different concentrations of iChs used in the course of the study are as seen in Table 6.1. The baseline concentration for each iCh was 48 µM, afterward concentrations of all the iChs was either reduced or increased exponentially.

Table 6.1: The amounts of dimethyl sulfoxide present in different concentrations of the novel iron-chelators used in the course of the study

71 (µM)	**Amount of DMSO (%)**	**75 (µM)**	**Amount of DMSO (%)**	**85 (µM)**	**Amount of DMSO (%)**	**70 (µM)**	**Amount of DMSO (%)**	**81 (µM)**	**Amount of DMSO (%)**
48	0.98	**48**	2.4	**48**	0.98	**48**	2.4	**48**	0
32	0.64	**32**	1.6	**64**	1.28	**64**	3.2	**64**	0
24	0.48	**24**	1.2	**96**	1.92	**96**	4.8	**96**	0
16	0.32	**16**	0.8	**126**	2.52	**126**	6.3	**126**	0
12	0.24	**12**	0.6	**192**	3.84	**192**	9.6	**192**	0
8	0.16	**8**	0.4	**256**	5.12	**256**	12.8	**256**	0
6	0.12	**6**	0.3	**300**	6	**300**	15	**300**	0
-	-	-	-	**350**	7	**350**	17.5	**350**	0
-	-	-	-	**400**	8	**400**	20	**400**	0

The chemical structures of the novel untested iron-chelators used in this study are as seen in Figure 6.1.

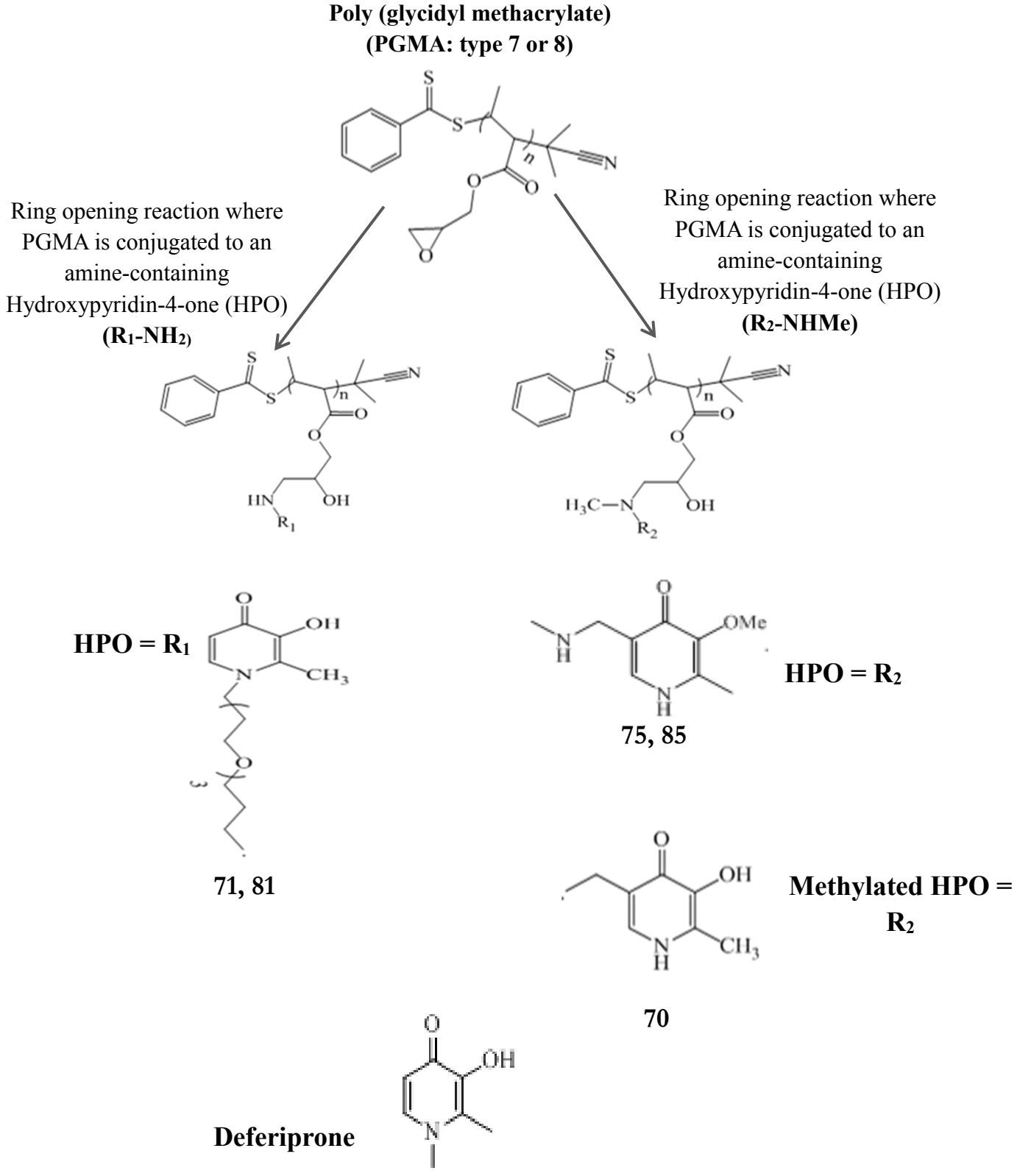


Figure 6.1: Structures of the novel iron-chelators used during the study. Iron-chelators 71, 75, 81, 70 and 85 were synthesized in ring opening reactions in which a PGMA was conjugated to an amine containing HPO (Modified from Li *et al*., 2016)

6.2.5. Antimicrobial assays

To assess the potential of the iChs to inhibit bacterial growth, microdilution antimicrobial assays were performed as described in section 2.2.6.2 (Chapter Two**)**. However, ~10^7 CFU/ml of bacteria were introduced per well in the absence or presence of different concentrations of the iChs under study. Controls that were set up include: (i) MRSA without iCh(s); and (ii) MRSA with different concentrations of DMSO. Plates were then incubated under aerobic conditions at 37ºC for 24 hrs. The number of viable bacteria in each well was determined by plating 20 µl (in duplicate) of the content of each well onto CLED agar. The numbers of colonies on agar were counted on a colony counter (Gallenkamp) after overnight incubation at 37ºC. The bacterial inhibition rate was calculated as follows:

$$(R = \frac{[X_0 - X_t]}{X_0} \times 100)$$

where R is the bacterial inhibition rate, X_0 the number of bacteria without iron-chelator (control), and X_t the number of bacteria following treatment with an iron-chelator.

6.2.6. Cytotoxicity assays

Cytotoxicity assays were performed to assess the cytotoxic effects of the novel iron-chelators as well as DMSO on KB epithelial cell line. Human KB epithelial cells were cultured and grown in 96-well plates in EMEM (~1x10^5 cells per 0.150 milliliter in each well) and incubated in a CO_2 incubator (95% humidity, 5% CO_2) at 37ºC till 80-100% confluence was attained. The growth medium was then discarded following which cells were washed with PBS to remove non-adherent cells. EMEM and the required concentrations of the iChs were then added to all wells as required. Only EMEM (100 µl/well) was added to designated high and low control wells while both DMSO (concentrations as stated in Table 5.1) and EMEM were added to DMSO control wells. Plates were subsequently incubated at 37ºC for 24hrs in a CO_2 incubator (95% humidity, 5% CO_2).

6.2.7. Measurement of lactate dehydrogenase

Following the incubations described in section 6.2.7, the toxic effects of the different variables on the KB epithelial cell line were determined in an LDH colorimetric assay as previously described in chapter four (section 4.2.5).

Percentage cytotoxicity was calculated as follows:

$$\left(\%\ Cytotoxicity = \frac{[Sample\ value - \ Low\ control\ value]}{[High\ control\ value - \ Low\ control\ value]} \times 100\right)$$

6.2.8. Statistical analysis

Unless otherwise stated every experiment was repeated 3 times and experimental data were analysed using a two-tailed paired t test (two-sample equal variance) for comparison between means. Values were considered statistically significant at a 95% confidence interval ($P < 0.05$).

6.3. Results

6.3.1. Antimicrobial effects of iron-chelators on a UK MRSA isolate

The antimicrobial effects of five novel iChs on a UK MRSA isolate (isolate 'Nasal') was assessed in this study. In consideration of the fact that limited quantities of each iCh was available and the fact that the antimicrobial potential of the iChs were unknown prior to this study, the collaborating chemists recommended that 48 µM of each iCh be used in antimicrobial assays to establish a baseline concentration for each iCh. This 48 µM concentration corresponds to 2.405mg/ml, 1.589mg/ml, 0.427mg/ml, 1.661mg/ml, 0.643mg/ml and 0.0067mg/ml for iChs 71, 75, 85, 70, 81 and deferiprone respectively.

At 48 µM, iron-chelators 71 and 75 were more effective at inhibiting bacterial growth than the other iron-chelators as they both had a bacterial inhibition rate of 100% (Figure 5.2).

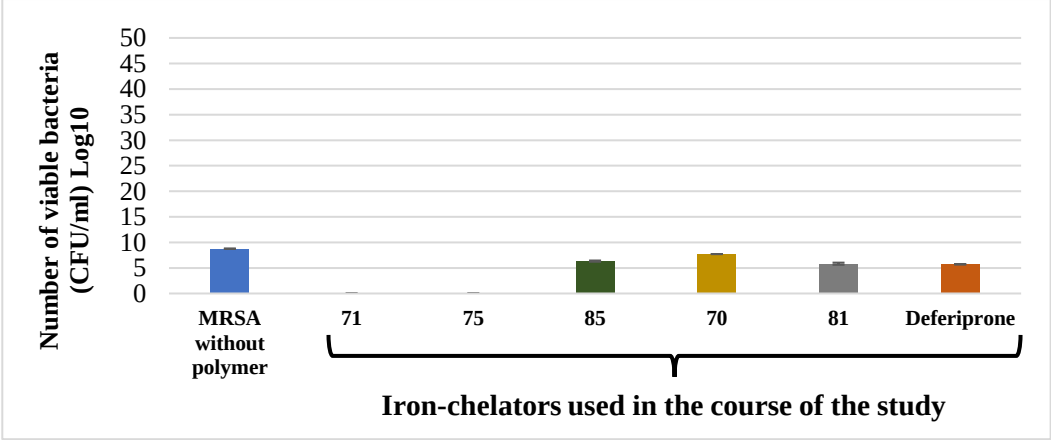


Figure 6.2: Antimicrobial effect of 48 µM of the iron-chelators on MRSA after 24 hr incubation. Results are the average of three independent experiments; error bars represent standard error.

Following on from the initial assay to assess antimicrobial effect of all the iChs at 48 µM, the lower concentrations of iChs 71 and 75 were used while higher concentrations of iChs 85, 70 and 81 were used in further analysis.

Although they both totally inhibited growth of bacteria at 48 µM, iCh 71 was seen to be more

effective than 75 as it totally inhibited bacterial growth at 12µM (0.601mg/ml) compared to 75 which totally inhibited growth at a higher concentration i.e. 24µM (0.795mg/ml) (Figure 5.3). Unlike iChs 71 and 75, Deferiprone still did not totally inhibit growth of MRSA at 48µM (0.048 mg/ml) (Figure 5.3). Concentrations of the iron-chelators 71, 75 and Deferiprone used in the course of the study are shown in Table 6.2.

Table 6.2: Concentrations of iron-chelators 71, 75 and Deferiprone used during the study

71 (mw=50,100)		**75 (mw=33,100)**		**Deferiprone (mw=139.152)**	
µM	mg/ml	µM	mg/ml	µM	mg/ml
6	0.301	6	0.199	**6**	0.0008
8	0.401	8	0.265	**8**	0.0011
12	0.601	12	0.397	**12**	0.0017
16	0.802	16	0.530	**16**	0.0022
24	1.202	24	0.794	**24**	0.0033
32	1.603	32	1.059	**32**	0.0045
48	2.405	48	1.589	**48**	0.0067

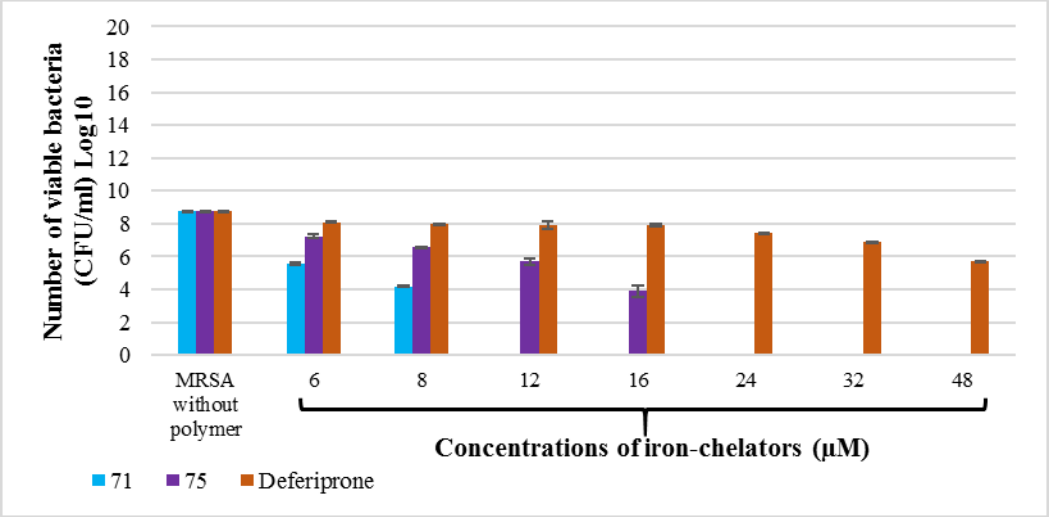


Figure 6.3: Antimicrobial effects of different concentrations of iron-chelators 71, 75 and Deferiprone on MRSA after 24 hr incubation. Results are the mean of three independent experiments; error bars represent standard error.

Results showed that DMSO did not significantly contribute to the observed antimicrobial effect of iCh 71 on MRSA. This is because the difference in the bacterial inhibition rate of iCh 71 (dissolved with 50% DMSO in water) and the bacterial inhibition rate of DMSO (present in the different concentrations of iCh 71) alone was significant based on the 2-tailed paired t test (*P* value = 2E-09) (Table 5.3A, Figure 5.4A). This was also the case with iCh 75 as the difference in the bacterial inhibition rate of iCh 75 (dissolved with 20% DMSO in water) and the bacterial inhibition rate of the DMSO (present in the different concentrations of iCh 75) alone was significant based on the 2-tailed paired t test (*P* value = 1.05E-06) (Table 6.3B, Figure 6.4B).

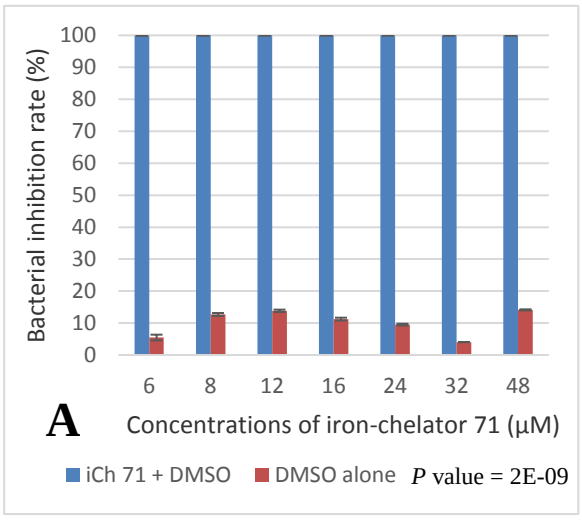
Table 6.3: Antimicrobial effects of different concentrations of iron-chelators 71 (dissolved with 50% DMSO in water) **(A)** and 75 (dissolved with 20% DMSO in water) **(B)** along with the antimicrobial effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on MRSA. Titles of series compared to obtain the *P* values are highlighted in green. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t test.

A

iCh 71				
iCh 71 (µM)	**Amount of DMSO present in each concentration (%)**	**Bacterial inhibition rates**		***P* value**
		iCh 71 + DMSO	**DMSO alone**	
6	0.12	99.986	5.476	2E-09
8	0.16	99.997	12.680	
12	0.24	100.000	13.833	
16	0.32	100.000	11.239	
24	0.48	100.000	9.510	
32	0.64	100.000	4.035	
48	0.98	100.000	14.121	

B

iCh 75				
iCh 75 (µM)	**Amount of DMSO present in each concentration (%)**	**Bacterial inhibition rates**		***P* value**
		iCh 75 + DMSO	**DMSO alone**	
6	0.3	96.772	11.239	1.05E-06
8	0.4	99.320	8.069	
12	0.6	99.894	4.035	
16	0.8	99.998	9.222	
24	1.2	100.000	23.631	
32	1.6	100.000	25.648	
48	2.4	100.000	34.582	



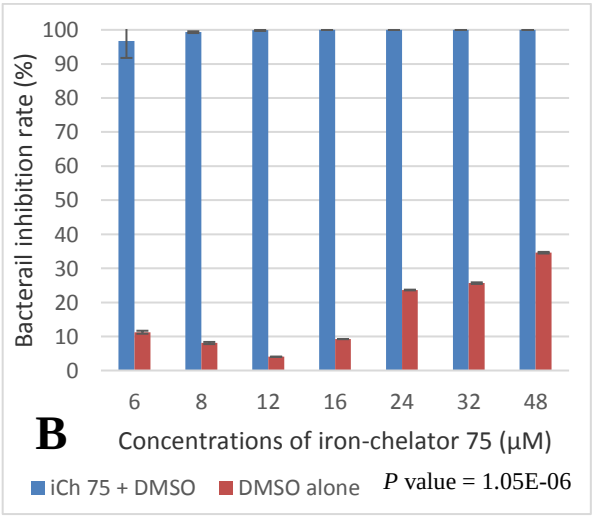


Figure 6.4: Antimicrobial effects of different concentrations of iron-chelators 71 (dissolved with 50% DMSO in water) **(A)** and 75 (dissolved with 20% DMSO in water) **(B)** along with the antimicrobial effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on MRSA. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t test.

as

the next most effective against MRSA after iCh 75. iCh 85 was less effective at inhibiting growth of MRSA than iCh 81 as it totally inhibited bacterial growth at a higher concentration of 400µM (3.560mg/ml) (Figure 6.5). iCh 70 had the least inhibitory effect on MRSA of all the novel iChs as total bacterial inhibition was seen at 126µM (4.360mg/ml) (Figure 6.5). On the other hand, the antimicrobial effect of deferiprone on MRSA was lower than the antimicrobial effect exerted by the novel iChs; at a concentration of 400µM (0.400mg/ml), deferiprone still did not totally inhibit bacterial growth (Figure 6.5). Concentrations of the iron-chelators 85, 70, 81 and Deferiprone used in the course of the study are shown in Table 6.4.

85 (mw=8900)		**70 (mw=34,600)**		**81 (mw=13,400)**		**Deferiprone (mw=139.152)**	
µM	mg/ml	µM	mg/ml	µM	mg/ml	µM	mg/ml
48	0.427	**48**	1.661	**48**	0.643	**48**	0.0067
64	0.570	**64**	2.214	**64**	0.858	**64**	0.0089
96	0.854	**96**	3.322	**96**	1.287	**96**	0.0134
126	1.121	**126**	4.360	**126**	1.688	**126**	0.0175
192	1.709	**192**	6.643	**192**	2.573	**192**	0.0267
256	2.278	**256**	8.858	**256**	3.430	**256**	0.0356
300	2.670	**300**	10.380	**300**	4.020	**300**	0.0417
350	3.115	**350**	12.110	**350**	4.690	**350**	0.0487
400	3.560	**400**	13.840	**400**	5.360	**400**	0.0557

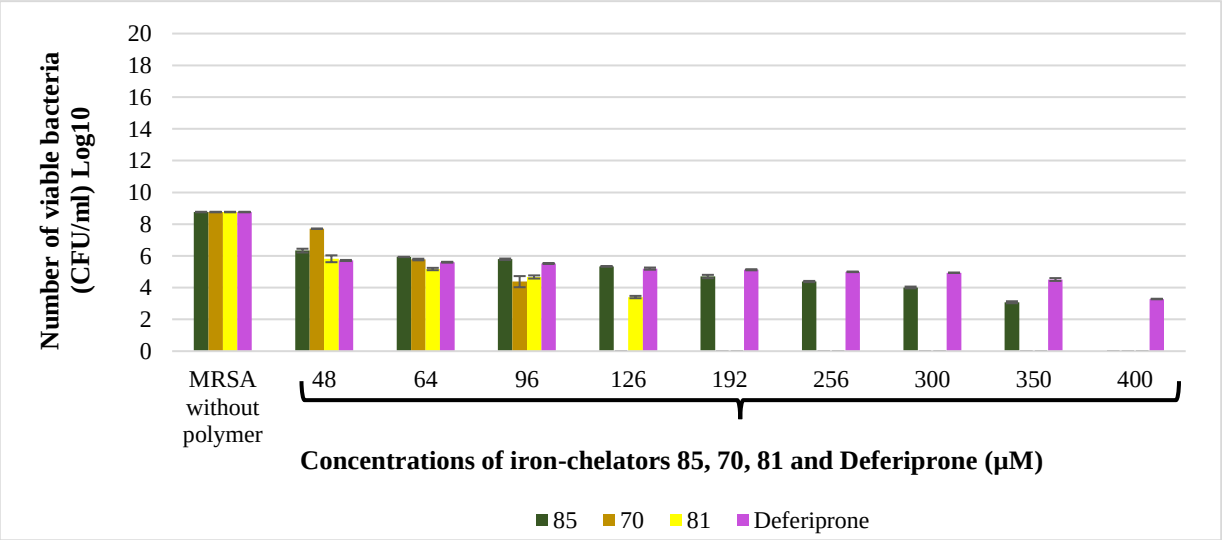


Figure 6.5: Antimicrobial effect of different concentrations of iron-chelators 85, 70, 81 and Deferiprone on MRSA after 24-hr incubation. Results are the average of three independent experiments; error bars represent standard error.

Once again, the results showed that DMSO did not significantly contribute to the observed antimicrobial effect of iCh 85 on MRSA. The difference observed between the bacterial inhibition rate of iCh 85 (dissolved with 50% DMSO in water) and the bacterial inhibition rate of the corresponding amount of DMSO (present in different concentrations of 85) alone on MRSA was significant based on the 2-tailed t test (*P* value = 0.0002) (Table 6.5A, Figure 6.6A). Furthermore, the results also showed that DMSO had no significant effect on the antimicrobial effect of iron-chelator 70. This is because the difference in the antimicrobial effect of iron-chelator 70 (dissolved with 20% DMSO in water) and the antimicrobial effect of the DMSO (present in the different concentrations of iron-chelator 70) alone on MRSA based on the 2-tailed t test was significant (*P* value = 0.015) (Table 6.5B, Figure 6.6B).

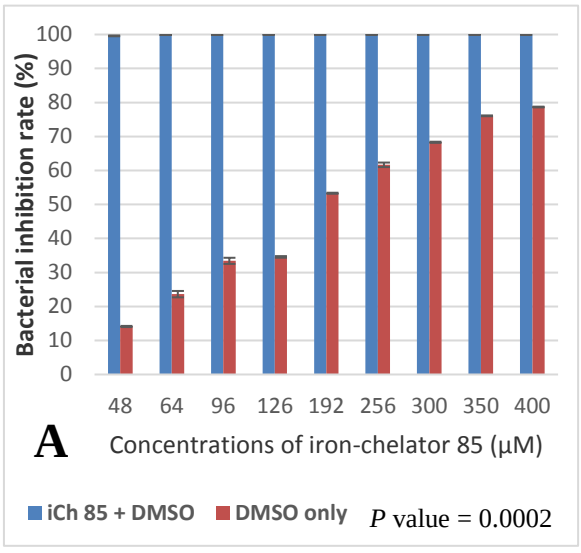
Table 6.5: Antimicrobial effects of different concentrations of iron-chelators 85 (dissolved with 50% DMSO in water) **(A)** and 70 (dissolved with 20% DMSO in water) **(B)** along with the antimicrobial effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on MRSA. Titles of series compared to obtain the *P* values are highlighted in green. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t test.

A

iCh 85				
iCh 85 (μM)	Amount of DMSO present in each concentration (%)	Bacterial inhibition rates		
		iCh 85 + DMSO	DMSO alone	*P* value
48	0.980	99.599	14.121	0.0002
64	1.280	99.860	23.631	
96	1.920	99.932	33.429	
126	2.520	99.990	34.582	
192	3.840	99.996	53.314	
256	5.120	99.999	61.671	
300	6.000	100.000	68.300	
350	7.000	100.000	76.081	
400	8.000	100.000	78.674	

B

iCh 70				
iCh 70 (μM)	Amount of DMSO present in each concentration (%)	Bacterial inhibition rates		
		iCh 70 + DMSO	DMSO alone	*P* value
48	2.400	91.124	34.582	0.015
64	3.200	99.896	45.245	
96	4.800	99.994	55.043	
126	6.300	100.000	68.300	
192	9.600	100.000	83.458	
256	12.800	100.000	87.435	
300	15.000	100.000	99.259	
350	17.500	100.000	99.973	
400	20.000	100.000	99.990	



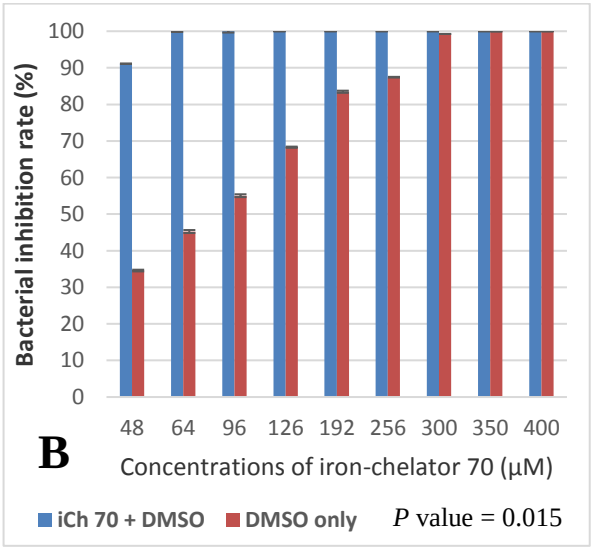


Figure 6.6: Antimicrobial effects of different concentrations of iron-chelators 85 (dissolved with 50% DMSO in water) **(A)** and 70 (dissolved with 20% DMSO in water) **(B)** along with the antimicrobial effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on MRSA. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t test

DMSO had no effect on the antimicrobial effect of iron-chelator 81 and deferiprone as they were both prepared by dissolving in water.

6.3.2. Cytotoxic effect of iron-chelators on KB epithelial cell line

The toxicity of iChs 71 and 75 on KB cells was evaluated in the 6 – 32 µM concentration range because the concentrations at which they totally inhibited growth of MRSA are below 48 µM. The toxicity of 75 was seen to be less than 50% at all concentrations within the evaluated concentration range unlike in the case of 71 where toxicity was always approximately equal to (8µM_49.65%) or greater than 50% (Figure 6.7). The toxic effect of deferiprone on KB cells within the 6 – 32 µM concentration range was seen to peak at 12 µM after which there was a reduction in the observed toxic effect at higher concentrations (Figure 6.7). The toxicity of deferiprone was seen to be < 15.00% at all concentrations within the evaluated concentration range.

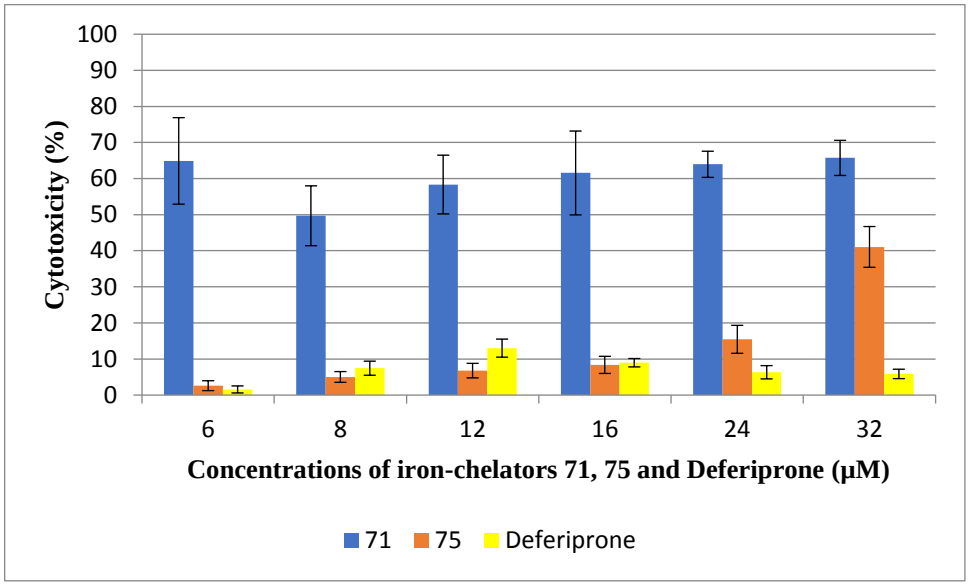


Figure 6.7: Cytotoxic effects of various concentrations of iron-chelators 71, 75 and Deferiprone on KB epithelial cell line after 24 hours. Results are the average of three independent experiments performed in duplicate; error bars represent standard error.

There was a significant difference between the cytotoxic effect of iCh 71 (dissolved with 50% DMSO in water) and the cytotoxic effect of the corresponding amount of DMSO (present in all concentrations of iCh 71) alone on KB epithelial cells based on the 2-tailed t-test (*P* value = 2.5E-06), which means that DMSO had no significant effect on the observed toxicity of iCh 71 (dissolved with 50% DMSO in water) on KB cells (Figure 6.8A). This was not the case with iron-chelator 75 as there was no significant difference between the toxic effect of iCh 75 (dissolved with 20% DMSO in water) and the cytotoxic effect of the corresponding amount of DMSO (present in all concentrations of iCh 75) alone on KB epithelial cells based on the 2-tailed t-test (*P* value = 0.098), which means that DMSO contributed to the observed toxicity of iCh 75 (dissolved with 20% DMSO in water) on KB epithelial cells (Figure 6.8B).

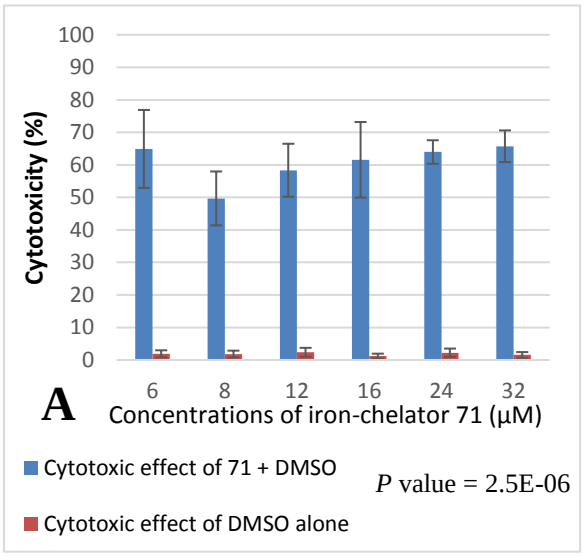
Table 6.6: Cytotoxic effects of different concentrations of iron-chelators 71 (dissolved with 50% DMSO in water) **(A)** and 75 (dissolved with 20% DMSO in water) **(B)** along with the cytotoxic effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on KB epithelial cells. Titles of series compared to obtain the *P* values are highlighted in green. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t-test.

A

iCh 71				
iCh 71 (µM)	**Amount of DMSO present in each concentration (%)**	**Cytotoxic effects**		
		iCh 71 + DMSO	**DMSO alone**	***P* value**
6	0.12	64.90	1.89	2.5E-06
8	0.16	49.68	1.82	
12	0.24	58.34	2.36	
16	0.32	61.55	1.23	
24	0.48	63.96	2.23	
32	0.64	65.72	1.54	

B

iCh 75				
iCh 75 (µM)	**Amount of DMSO present in each concentration (%)**	**Cytotoxic effects**		
		iCh 75 + DMSO	**DMSO alone**	***P* value**
6	0.3	2.60	1.23	0.098
8	0.4	5.02	1.70	
12	0.6	6.77	1.54	
16	0.8	8.37	2.07	
24	1.2	15.45	3.62	
32	1.6	41.06	5.29	



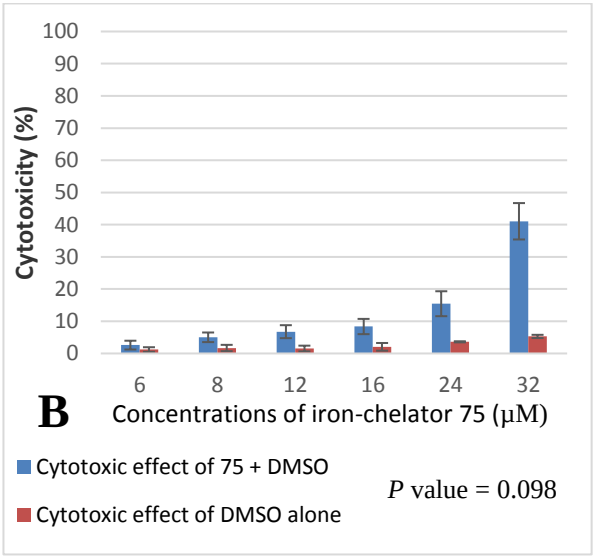


Figure 6.8: Cytotoxic effects of different concentrations of iron-chelators 71 (dissolved with 50% DMSO in water) **(A)** and 75 (dissolved with 20% DMSO in water) **(B)** along with the cytotoxic effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on KB epithelial cells. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t test.

As the concentrations at which they totally inhibited growth of MRSA are higher than 48 µM, the cytotoxic effects of iChs 85, 70 and 81 on KB epithelial cells were evaluated within the 48-350 µM concentration range. iCh 70 was seen to be the most toxic of the three iron-chelators on KB cells within the evaluated concentration range followed by 85 and then 81 which was the least toxic of the three (Figure 6.9). The cytotoxic effects of all three of them i.e. iChs 85, 70 and 81, peaked at concentration 256 µM (i.e. 50.87%, 70.01% and 37.85% respectively) after which a decline in toxic effects was observed. Within the 48-350 µM concentration range, deferiprone was the most toxic on KB cells after iCh 70 up till concentration 256 µM; at higher concentrations, deferiprone was seen to be the most toxic on KB cells (Figure 6.9).

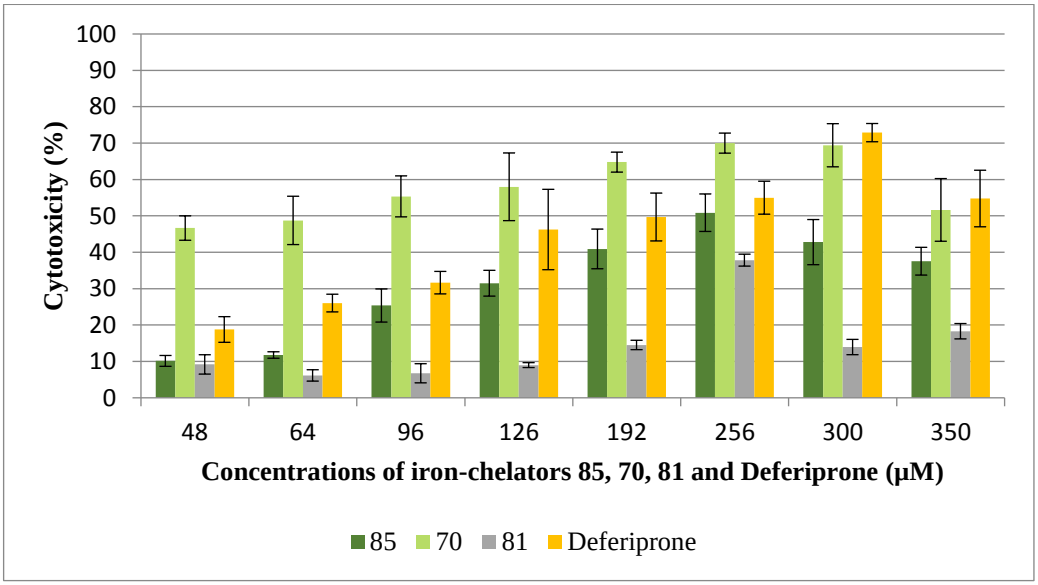


Figure 6.9: Cytotoxic effects of different concentrations of iron-chelators 85, 70, 81 and Deferiprone on KB epithelial cell line after 24 hours. Results are the average of three independent experiments performed in duplicate; error bars represent standard error.

DMSO did not significantly contribute to the observed toxic effect of iCh 85 (dissolved with 50% DMSO in water) on KB epithelial cell line. There was significant difference between the toxicity of DMSO (present in all concentrations of iCh 85) alone and the toxicity of iCh 85 (dissolved with 50% DMSO in water) on KB epithelial cells based on the 2-tailed t-test (*P* value = 0.005) (Table 6.7A, Figure 6.10A). This was also the case with iCh 70 as a significant difference was observed between the toxic effect of DMSO (present in all concentrations of iCh 70) and the toxic effect of iCh 70 (dissolved with 20% DMSO dissolved in water) on KB epithelial cells based on the 2-tailed t-test (*P* value = 8.37E-05) (Table 6.7B, Figure 6.10B).

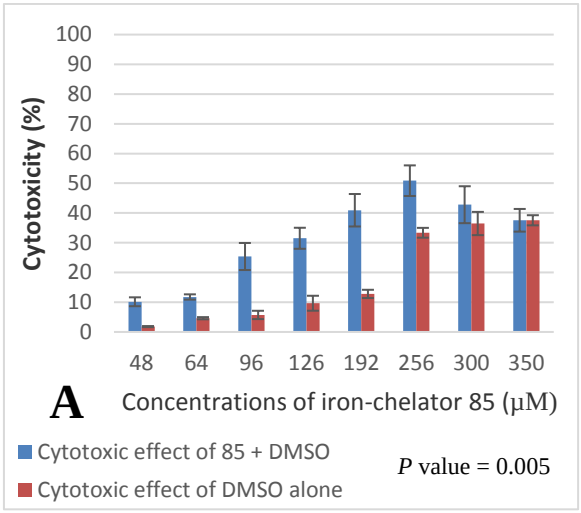
Table 6.7: Cytotoxic effects of different concentrations of iron-chelators 85 (dissolved with 50% DMSO in water) **(A)** and 70 (dissolved with 20% DMSO in water) **(B)** along with the cytotoxic effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on KB epithelial cells. The series compared to obtain the *P* values are highlighted in green. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t-test.

A

iCh 85				
iCh 85 (µM)	Amount of DMSO present in each concentration (%)	Cytotoxic effects		P value
		iCh 85 + DMSO	DMSO alone	
48	0.980	10.15	1.80	0.005
64	1.280	11.76	4.61	
96	1.920	25.37	5.72	
126	2.520	31.50	9.65	
192	3.840	40.93	12.79	
256	5.120	50.87	33.35	
300	6.000	42.79	36.46	
350	7.000	37.55	37.55	

B

h 70				
iCh 70 (µM)	Amount of DMSO present in each concentration (%)	Cytotoxic effects		P value
		iCh 70 + DMSO	DMSO alone	
48	2.400	46.66	9.65	8.37E-05
64	3.200	48.76	12.56	
96	4.800	55.36	33.35	
126	6.300	57.99	36.46	
192	9.600	64.79	12.79	
256	12.800	70.01	47.87	
300	15.000	69.41	38.61	
350	17.500	51.65	27.75	



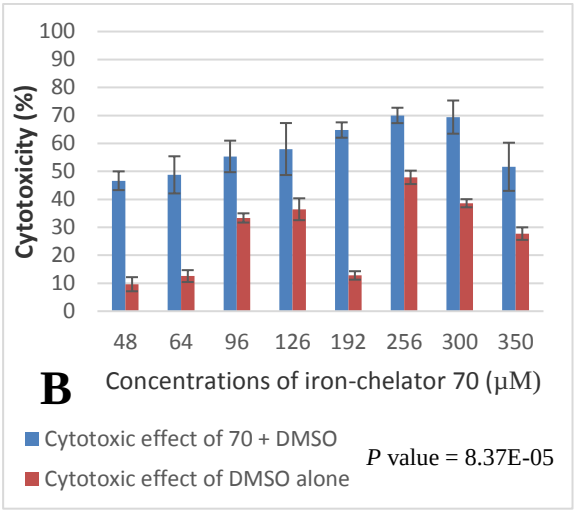


Figure 6.10: Cytotoxic effects of different concentrations of iron-chelators 85 (dissolved with 50% DMSO in water) **(A)** and 70 (dissolved with 20% DMSO in water) **(B)** along with the cytotoxic effects of the corresponding amounts of DMSO present in every concentration of both iron-chelators on KB epithelial cells. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed paired t-test.

DMSO had no effect on the cytotoxic effects of iChs 81 and Deferiprone as they were both prepared by dissolving in water.

Table 6.8: Summary of the characteristics of all the iron-chelators used in this study

Iron-Chelator	Molecular Weight (Da)	pFe^{3+}	3-Hydroxypyridin-4-one (HPO)	Poly(glycidyl methacrylate)(PGMA)
71	50,100	19.47	1	7
75	33,100	15.9	5	7
70	34,600	-	5b	7
81	13,900	19.47	1	8
85	8,900	15.9	5	8
Deferiprone	139.152	20.5		None

6.4. Discussion

The antimicrobial effects of iChs 71, 75, 81, 85, 70 and deferiprone on MRSA, as well as their toxic effects on KB epithelial cell line were evaluated during this study. Similar to other studies that showed that a strong dependence existed between the molecular weight (mw) of antimicrobial polymers and their antimicrobial action against *S. aureus* (Ikeda and Tazuke, 1983; Ikeda *et al*., 1986; Kanazawa *et al*., 1993; Kenawy *et al*., 2007), results from this study showed that the inhibitory effect of the iChs on MRSA is dependent on their molecular weights. Amongst the novel iChs, iCh 71 was the most effective at inhibiting MRSA followed by iChs 75 (Figure 6.3), 81 and 85 respectively (Figure 6.5); their corresponding molecular weights in that order are 50100, 33100, 13400 and 8900 Da. In addition, apart from iCh 70, all the novel iChs with PGMA type 7 had higher mws and a greater inhibitory effect on MRSA than their counterparts with PGMA type 8. In 2009, Kim and Shin previously demonstrated that deferiprone can inhibit the growth of staphylococci including *S. aureus*. Similarly, results from this study have shown that deferiprone inhibits the growth of MRSA; however, as it had the lowest mw (139.152 Da) of all the studied iChs, it exerted the least inhibitory effect on MRSA i.e. even at 400µM (0.0557mg/ml), it did not totally inhibit the growth of MRSA (Figures 6.3 and 6.5).

Although the mw of iCh 70 was 33,100 Da, which is higher than the mws of chelators 81 and 85, it was the least effective of the novel chelators at inhibiting growth of MRSA (i.e. at 126µM_4.360mg/ml; Figure 6.5). This is possibly because methylation of a hydroxyl group present on the HPO units of iCh 70 interferes with iron chelation. Thus, the inhibitory effect of the iChs on MRSA can be said to also depend on the iron-chelating mechanism of the iChs. Chelators with high pFe^{3+} have been predicted to scavenge iron more effectively at low concentrations (Hider *et al*., 2000) and in this study, iChs with HPO type 1 (pFe^{3+} = 19.47) were seen to totally inhibit MRSA at lower concentrations than their counterparts with HPO 5 (pFe^{3+} = 15.9) and 5b. iCh 71

totally inhibited MRSA at a lower concentration (at 12µM_0.601mg/ml; Figure 6.3) than its counterpart with the same PGMA type i.e. iCh 75 (at 24µM_0.795mg/ml; Figure 5.3). Similarly, iCh 81 totally inhibited MRSA at a lower concentration (at 192µM_2.573mg/ml; Figure 6.5) than its counterpart with the same PGMA type i.e. iCh 85 (at 400µM_3.560mg/ml; Figure 6.5). Deferiprone has a pFe^{3+} of 20.5 yet it did not inhibit 99% of bacterial growth within the concentration range (6µM_0.0008mg/ml - 400µM_0.0557mg/ml) used in this study (Figure 6.3 and 6.5). Thus, the greater mws and the presence of the PGMA component in the novel iChs may be the reason for their greater inhibitory effect exerted on MRSA compared with deferiprone.

DMSO is the most widely used solvent for the solubilisation of large chemical libraries (Manner *et al*., 2013) because it is partly soluble in both organic and aqueous media (Da Violante, 2002). It interacts with the metabolism and membranes of cells thus resulting in severe cell damage (Da Violante 2002). Several studies, (e.g. Basch and Gadebusch, 1968; Wadhwani, 2008) have previously demonstrated that DMSO has antimicrobial potential. In the present study, evaluation of the inhibitory effect of DMSO alone on MRSA showed that DMSO indeed inhibited growth of MRSA as seen in Figures 6.4 and 6.6. A comparison of the inhibitory effect of DMSO alone with the inhibitory effect of the iron-chelators (prepared by dissolving with either 50% or 20% of DMSO in water i.e. iChs 71, 85, 75 and 70) on MRSA, showed that DMSO did not significantly contribute to the observed inhibitory effects of any of the iChs, used in this study, on MRSA, had a significant effect on the inhibitory effect of iron-chelator 85 alone which means that DMSO enhanced the inhibitory effect of iron-chelator 85 on MRSA. This finding contrasts with the findings by Tarrand *et al*., 2012 who have previously shown that DMSO enhanced the effectiveness of skin antiseptics (Tarrand *et al.,* 2012).

With regards the novel iChs, iCh 71 (mw=50,100Da) was seen to be more toxic on KB cells compared with iCh 75 (mw=33,100Da) within the same concentration range of 6-32 µM. Also,

within the concentration range of 48-350 µM, iCh 70 (mw=33,100Da) was seen to be the most toxic on KB cells followed by 85 (mw=8900Da) and 81 (mw=13,400Da) respectively. These results are similar to findings in the study by Struck *et al*., 2008, where they showed that there is a correlation between the molecular weight of compounds their toxic effect on different cancer cell lines. However, deferiprone which has a mw of 139.152 Da was seen to be more toxic than the higher mw iChs 85 (8900 Da) and 81 (13,400 Da) (both of which bear the PGMA type 8) on KB cells. Thus, the component(s) of iChs also have a role to play in cytotoxicity.

DMSO is a widely used cryoprotectant (Chaytor *et al*., 2012) and the amount of DMSO suitable for use with cells, i.e. amount of DMSO that will not damage membrane integrity of cells, varies for different cell types (Da Violante, 2002). Studies such as those by Julien *et al*., 2012 and Hanslick *et al*., 2009, have previously demonstrated that DMSO can be toxic on mammalian cells both *in vivo* and *in vitro*. Figures 6.8 and 6.10 showed that DMSO exerts a toxic effect on KB epithelial cell line. Absence of DMSO in the solvent used to prepare iCh 81 may be the reason why chelator 85 was more toxic on KB cells than iCh 81 (Figure 6.9) regardless of the fact that iCh 81 had a higher mw. Comparison of the toxic effects of DMSO alone and the toxic effects of the iChs (prepared by dissolving with either 50% or 20% of DMSO in water i.e. iChs 71, 85, 75 and 70) on KB epithelial cells showed that DMSO contributed significantly to the observed toxic effects of iron-chelators 75 on KB cells while it did not contribute significantly to the observed toxic effect of iron-chelator 71, 85 and 70 on KB cells.

Some strategies used for the design of iron-chelators, such as that which involves the immobilisation of natural chelators onto activated supports, have the limitation of being expensive. However, the process of synthesising HPO-functionalized iron chelating homopolymers in ring-opening reaction of RAFT-based PGMA using HPOs with various amine-substituents, as described in Li *et al*., 2016, has proven to be a simple technique with high yield and low cost. The observed

anti-MRSA potential of the synthesised iChs, as shown from the results in this study, makes up part of the work published in Li *et al.*, 2016. These results show the functionality of the novel iChs, used in this study, as potential anti-MRSA agents. Considering the limitation in therapeutic options available for treatment of MRSA infections as well as the stall in the development of new antibiotics by the pharmaceutical industry, identification of these novel agents with anti-MRSA functionality potentially provides an alternative to the currently increasingly unsuccessful conventional agents used for the treatment of MRSA (i.e. antibiotics).

Therefore, the RAFT polymerisation approach for the synthesis of iChs as used in this study can be applied to the syntheses of other related types of functional materials with antimicrobial potential.

Chapter Seven

General Discussion

7.1. Overview

Staphylococcus aureus is a major human pathogen (Laabei *et al*., 2014; Tong *et al*., 2015; Rong *et al*., 2017) that can cause a broad variety of serious infections, perhaps even more than any other bacterial species (Moellering, 2012). *Staphylococcus aureus* has proven to be one of the most persistent pathogens in the healthcare and community settings (Deleo and Chambers, 2009; Hamdan-Partida *et al*., 2010; Uhlemann *et al*., 2014). The ability of *S. aureus* to be such a capable pathogen while also appearing as part of the normal flora of humans can largely be attributed to the myriad of cell surface and secreted virulence factors that it produces (Spaudling *et al*., 2013). It displays a wider variety of virulence mechanisms than virtually any other human pathogen and is a model for the study of the pathogenesis of infectious diseases (Archer, 1998; Chauhan and Varma, 2009). These virulence determinants facilitate tissue adhesion, immune evasion as well as host injury (Kong *et al.,* 2016). Emergence of MRSA strains resistant to β-lactam antibiotics has further complicated the treatment of *S. aureus* infections (French, 2006). Expression of PBP2A, encoded by the *mecA* gene, with low affinity for β-lactams is the key determinant for β-lactam resistance (Reichmann and Pinho, 2017).

Methicillin resistant *S. aureus* has acquired resistance to multiple antibiotic classes (tetracyclines, macrolides, streptogramins, lincosamides, aminiglycosides) and this has limited the therapeutic options available for treatment of MRSA infections (Loffler *et al*., 2010; Chatterjee and Otto, 2013). To further complicate this, MRSA has the capacity to acquire new exogenous genes (Moellering, 2011) which may have the potential to confer resistance to anyone of the few antibiotics currently available to treat MRSA infections. Though development of such resistance does not cause MRSA to be more intrinsically virulent, resistance makes MRSA infections more difficult to treat and hence more dangerous (Kong *et al*., 2016). The relatively minor fitness costs associated with the resistance of MRSA to methicillin and other β-lactam antibiotics has resulted in high infiltration of hospital and community settings (Li *et al*., 2012).

The rise in the incidence of drug resistant *S. aureus* strains over the past few decades has led to efforts to identify novel strategies in addition to novel antibiotics in a bid to combat infections (Reichmann and Pinho, 2017). Economic and regulatory obstacles have however stalled the development of new antibiotics by the pharmaceutical industry (Ventola, 2015). This lack of novel antimicrobial agents in the era of an increasingly dry drug development pipeline as well as a continued emergence of resistance among several pathogenic bacteria species continues to pose a challenge to clinicians (Howden, 2014; Kali, 2015). Consequently, clinicians and scientists have been exploring various means of using previously discarded agents and are also trying to use well established agents in new ways (Howden, 2014). Given this state of MRSA therapy and antibiotic development, it would seem that there is an urgent need to explore non-antibiotic options for the treatment of MRSA infections. Similar to studies that have in previous times demonstrated the antimicrobial potential of iron-chelators (Qiu *et al*., 2011; Thompson *et al*., 2012; Zhou *et al*., 2014), this study has demonstrated the anti-MRSA capacity of five novel iron-chelators that were synthesised using the RAFT polymerisation technique. However, the high toxic effect of these iron-chelators on the mammalian cell line (KB epithelial), as observed in this study, may constitute a limiting factor to their potential for use in clinical applications.

Diagnostic testing is essential to the containment of antimicrobial resistance (Okeke, 2016). The judicious use of appropriate tests, within the context of an effective laboratory system, promotes targeted and rational use of antimicrobials thereby lowering selective pressure for resistance (Okeke, 2016). Furthermore, diagnostic testing lowers healthcare costs and can identify treatment failure due to resistance; as a consequence, the nature and extent of resistance is unveiled and spread of resistance is prevented (Okeke, 2016). Two techniques commonly used in clinical laboratories to test antimicrobial susceptibility are the MIC and antibiotic disc diffusion techniques. Dilution methods used to determine the MICs of antimicrobial agents are the reference method for antimicrobial susceptibility testing-AST (EUCAST, 2003). Nevertheless, the disc

diffusion test remains one of the most widely used antimicrobial susceptibility testing methods in routine clinical microbiology laboratories (Matuschek *et al*., 2014).

Both the disc diffusion and MIC AST methods were used to assess the antibiotic susceptibility profiles of all the MRSA isolates (UK and Nigeria) under study. Results from the disc susceptibility tests showed that nine (56.25%) of the 16 Nigerian MRSA used in the course of this work were resistant to at least six of the 12 antibiotics that were studied (Section 2.3.5, Table 2.4). Contrarily, none of the UK isolates were resistant to ≥ 5 of the antibiotics under study. In the same vein, results showed that the MICs of Tetracycline, Vancomycin and Gentamicin were higher for a higher proportion of Nigerian MRSA compared with the UK MRSA (Tables 2.6, 2.7 and 2.9 respectively). This higher rate of resistance observed among Nigerian MRSA may be attributed to several factors that constitute a challenge to the Nigerian healthcare system, i.e. availability of antibiotics without prescription, misuse of antibiotics by physicians in clinical practice, availability of poor quality antibiotics, inadequate hospital infection control practices (Okeke *et al*., 1999; Kimanga 2012; Igbeneghu 2013) as well as circulation of counterfeit/substandard antibiotics, (Nasir *et al*., 2015).

Success of modern medical interventions at all levels of health care is threatened by the loss of antibiotic effectiveness (Grundmann, 2014), thus the need for effective global surveillance. Surveillance is defined as the generation and timely provision of information to inform decision making and action (Johnson, 2015). The essential core data for surveillance of antibiotic resistance (ABR) is generated by microbiology laboratories that routinely identify and ascertain the antibiotic susceptibility profiles of bacteria isolated from clinical specimens (Johnson, 2015). Yet like other developing countries, laboratories are perhaps the most neglected of all hospital departments in Nigeria (Nasir *et al*., 2015). Gathering of ABR data in Nigeria is hampered by the lack of a comprehensive policy and plan to address ABR, weak laboratory capacity on ABR testing and reporting and lack of ABR surveillance strategies (Nasir *et al*., 2015; Abubakar and Sulaiman,

2018). Unlike in Nigeria, the mainstay of infectious disease surveillance in the UK is the voluntary reporting of microbiological diagnoses by hospital laboratories to public health England and its predecessors (the Health Protection Agency and the Public Health Laboratory Service) (Johnson, 2015). Outputs from this surveillance system have focused on national trends in resistance in common pathogens e.g. *S. aureus* (Johnson, 2015). This variation in the frameworks of surveillance of ABR in Nigeria and UK may also account for the variations in the antibiotic susceptibility profiles exhibited by the UK and Nigerian MRSA under study. It also emphasises the need for a formal framework for collaboration among surveillance programs worldwide. Furthermore, to tackle the challenges associated with ABR surveillance in Nigeria, there is an urgent need for the development of high quality accredited medical/pathology laboratories in both the public and private sectors. An efficient system should also be put in place for the national co-ordination of ABR data generated from these laboratories.

Data about the epidemiology and prevalence of staphylococcal infections in Africa are scarce compared to information about such infections in the rest of the world (Okon *et al*., 2009). Nevertheless, the burden of *S. aureus* infection in Africa is reported to be high; but like other major bacterial pathogens, it is not yet considered in future research agendas (Schaumburg *et al*., 2014). Furthermore, loose links to global markets means interest in the dissemination of emerging pandemic pathogens might have been low in Africa (Schaumburg *et al*., 2014). Moreover, in addition to inadequate financing and shortages in infrastructure, the healthcare system in Africa is highly burdened by widespread communicable (e.g. HIV, tuberculosis) and non-communicable diseases (e.g. malaria) (Falagas *et al*., 2013). Consequently, detailed molecular characterisation of clinical *S. aureus* isolates from Africa has been largely neglected; only cases of *S. aureus* infections in travellers returning from Africa have suggested that *S. aureus* isolates from Africa might have a different genetic background and might be more virulent than isolates from Europe (Schaumburg *et al*., 2014). Like other African countries, several studies have shown that *S. aureus* is among the

most frequently encountered microorganisms in microbiology laboratories in Nigeria (Ako-Nai *et al*., 1995; Oliveira *et al*., 2001; Adejuyigbe *et al*., 2001; Ako-Nai *et al*., 2002; Ambe *et al*., 2007; Obidike *et al*., 2009; Ubani, 2009), yet, data on the molecular epidemiology of this pathogen in Nigeria is very limited (Shittu *et al*., 2011).

Some molecular characterisation of the MRSA isolates used in this study was done i.e. PCRs to confirm the identities of the isolates, classify the isolates (into clonal types), identify virulence factors and type of SCC*mec* element borne by the isolates. The results showed that each isolate bore at least six (46.15%) of the 13 virulence factors investigated. Distribution of some virulence factors in *S. aureus* is related to clonal type, whereas the presence of others is unrelated to genetic background (Peacock *et al*., 2002). In agreement with this, results from this study showed that all the MRSA isolates in the ST239 lineage (Nigerian isolates 114, 193, 176, 177, 162, 105, 112, UM44, 113, 158 and 178) have the genes that encode cytotoxins *hlb* and PSMα, as well as those that encode the surface proteins *fnbA* and *fnbB* in common (Table E.3, Appendix E). Although the distribution of MRSA clones in Africa is not well described (Abdulgader *et al*., 2015), the ST239/241-MRSA-I/III/IV clone has been reported to be prevalent on the whole African continent (Schaumburg *et al*., 2014). Findings from this study confirm this statement as 11 (68.75%) of the 16 Nigerian MRSA isolates belong to the ST239 lineage, while 9 (81.82%) of these 11 isolates bear the SCC*mec* type III element.

Presence or absence of the 13 virulence factors investigated during this study (Section 2.3.3, Table 2.3) may have in one way or another played a role in the observed cytotoxic effects of MRSA (live, heat killed, conditioned media) on the mammalian cell lines (KB and A549). For instance, the α-toxin is known to be an important inducer of toxicity (Prince *et al*., 2012; Loffler *et al*., 2014), however, results presented in chapter two (Table 2.3, Table E.5 (Appendix E)) showed that all (100%) the UK MRSA and only 5 (62.5%) Nigerian MRSA used in the cytotoxicity experiment bear the α-toxin gene. Yet, results from the cytotoxicity assays showed that the means of the toxic

effects of live Nigerian MRSA were higher than those of their UK counterparts on the cell lines 6 and 24 hrs post-infection at both MOIs (10:1 and 100:1). Therefore, the toxicity induced by each isolate cannot be wholly attributed to the α-toxin and differences in the toxicities induced by the UK and Nigerian MRSA isolates cannot be based on the presence or absence of the α-toxin. Results from the characterisation of the isolates (Section 2.3.3, Table 2.3) further showed that the sphingomyelinase β-toxin is present in all the Nigerian isolates but in only 50% of the UK isolates. Also, 100% of the Nigerian isolates and only 66.67% of the UK isolates bear the γ-haemolysin (*hlγ*) (Section 2.3.3, Table 2.3, Table E.5 (Appendix E)). Thus, the *hlb* and *hlγ* genes probably played more prominent roles than the α-toxin in the observed differences in toxicity between the UK and Nigerian isolates. The gene for δ toxin was amplified from the genomes of all six UK and only six Nigerian MRSA used in the cytotoxicity assays (Table 2.3). Notwithstanding, the two isolates that did not bear this gene (Nigerian isolates '114' and '162') were sometimes more toxic on cells than those that bear the *hlδ* gene (Sections 4.3.1.1 and 4.3.1.2). In addition, all the MRSA isolates involved in this study bear the gene for the PSMα toxin (Section 2.3.3, Table 2.3), a known mediator of phagolysosomal escape. Findings from the cytotoxicity assays (Tables 4.1 – 4.8) showed that, regardless of their genetic background, all the live MRSA exerted varying levels of toxicity on both KB and A549 cells; this did not however explain why some isolates were more toxic than others.

Therefore, findings from this study point to the possibility that differences in the toxic effects exerted by MRSA isolates (UK and Nigerian), at least on the cell lines used in the course of this work, is not due to a single virulence factor. Rather it is most likely a result of synergistic activity between the α-toxin, β-toxin, δ-toxin, γ-haemolysin, PSMα and other virulence factors. This is similar to findings from the study by Kumar and Kumar, 2015; they investigated the role of individual virulence factors (i.e. peptidoglycan, lipoteichoic acid, staphylococcal protein A, α-toxin, and Toxic-shock syndrome toxin 1) in the pathogenesis of staphylococcal endophthalmitis

and found that the effect of any one virulence factor was not sufficient to cause endophthalmitis. Rather, they found that different *S. aureus* virulence factors incited differential innate responses in the retina. Furthermore, the means of the cytotoxic effects of the CM of the Nigerian isolates on the cell lines were not significantly higher than those of their UK counterparts under all conditions (Tables 4.1 – 4.3, Tables 4.5 – 4.8) except 24 hrs post-infection at MOI 100:1 on KB cells (Table 4.4). This finding is an indication of the close similarity in the virulence repertoire of both the UK and Nigerian MRSA isolates. Therefore, there is a need for more rigorous molecular study of the UK and Nigerian MRSA to obtain conclusive answers regarding the differences in their virulence potentials.

In addition to the ability to invade and survive within epithelial and endothelial cells, *S. aureus* can also induce apoptosis in these cells following internalisation (Tucker *et al*., 2000; Garciarena *et al*., 2015). Although it has previously been shown that UV inactivated *S. aureus* are internalised by host cells, they did not induce apoptosis in the host cells (Mezies and Kourteva 1998), thus indicating that metabolically active internalised staphylococci are required for the induction of apoptosis (Esen *et al*., 2001; Loffler *at al*., 2005). Based on these, interactions of live MRSA (UK and Nigerian) with mammalian cells as well as the cytotoxic effects of the live MRSA on mammalian cells were assessed in the course of this work. Findings from this study showed that both the UK and Nigerian MRSA isolates invaded the mammalian cell lines at varying levels (KB and A549) (Sections 3.3.1.2 and 3.3.2.2). However, no significant differences were observed in the level at which the UK and Nigerian MRSA isolates invaded the mammalian cell lines (Figures 3.2 and 3.7; Tables 3.2 and 3.7). Furthermore, results obtained also showed that metabolically active *S. aureus* indeed induce apoptosis because flow cytometry aided the identification of apoptotic host cells 6 and 24 hrs post-MRSA infection (Section 4.3.2). Results from the flow cytometry experiments gave a breakdown of the distribution of apoptotic and necrotic host cells

following invasion of live MRSA on the mammalian cells lines, and these results showed that majority of the infected host cells were apoptotic (early or late stage) (Tables 4.9 - 4.12).

Findings from this study point to the possibility of African *S. aureus* (represented by Nigerian MRSA) indeed having a different genetic background than those from other parts of the world (represented by UK MRSA). Therefore, health organisations across Africa need to devote resources to the collaborative study of the epidemiology of *S. aureus* on the African continent. The Nigerian government need to also allocate resources to set up an efficient system for the surveillance and reporting of *S. aureus* (particularly MRSA) infections so as to avert disaster in times of epidemic and pandemic outbreak of *S. aureus* infection.

7.2. Limitations of the study

The primary aim of this study was to compare the virulence potential of both UK and Nigerian MRSA isolates. Thus, representative clinical MRSA isolates were obtained from both countries. Unfortunately, even though it is known that a larger sample size increases precision of the study finding and reduces the size of the sampling error, a major setback of this study was that only 22 MRSA isolates (six UK and 16 Nigerian) were used during this research.

Another limitation with regards the isolates is the limited geographical regions from which isolates from both countries were obtained. While the UK isolates were obtained only from Colchester, the Nigerian isolates were obtained from only one (North-eastern region) of the six geopolitical regions in Nigeria.

Methicillin resistant *S. aureus* are known to express a vast array of virulence factors and with the aid of PCR, presence of some of those virulence factors were verified in the MRSA isolates studied. Findings from this study also showed that the live Nigerian MRSA isolates exerted a higher cytotoxic effect on the KB and A549 mammalian cell lines. Unfortunately, though several virulence factors known to play a role in the cytotoxic effect of MRSA were identified in both the

UK and Nigerian isolates, time constraints hindered the identification of the precise virulence factor(s) responsible for the observed differences in cytotoxic effects.

Biofilm formation in microtiter plates, as used in this study, is the method most commonly used to screen for biofilm formation capacity (Azeredo *et al*., 2017). This technique has the advantages of being inexpensive, not needing advanced equipment apart from plate reader, and its potential for application in high throughput research (Azeredo *et al*., 2017). However, this technique is limited by the fact that it is unsuitable for investigating early stages of biofilm formation, it only takes end point measurement; parts of the biomass may stem from cells sedimented to the bottom of the wells, and the fact that loosely attached biofilm may not be measured correctly (can be detached during washing steps) (Azeredo *et al*., 2017).

One limitation common to the techniques used in the measurement of cell death in this study i.e. LDH and apoptosis/necrosis assays, is that they failed to provide real-time continuous monitoring of cell viability. Instead, they measured only a single endpoint parameter i.e. LDH activity following release from cells with altered cell membranes (LDH assay) and PS exposure (apoptosis/necrosis assay). The problem with using a single morphological or biochemical feature as an indicator of cell death is that most phenomena that are associated with cell death can also occur in cell death-unrelated scenarios; these features such as caspase activation, the exposure of PS on the outer leaflet of the plasma membrane etc., also constitute cytoprotective response to stress (Kepp *et al*., 2011).

Cancer cell lines, which are often relatively resistant to cell death induction, are highly heterogeneous, owing to the genetic and epigenetic alterations affecting cell death-regulatory molecules (Hanahan and Weinberg 2000; Kepp *et al*., 2011). Therefore, some of the biochemical processes that are routinely monitored to quantify cell death may not occur, or they may occur with very different kinetics in various experimental settings (Kepp *et al*., 2011). For example,

autophagy-deficient cells fail to expose phosphatidylserine during apoptosis (Qu *et al*., 2007; Kepp *et al*., 2011) and mouse embryonic fibroblasts lacking the pro-apoptotic BCL-2 family members BAX and BAK1 die in response to chemotherapy without manifesting the morphological and biochemical signs of apoptosis (Shimizu *et al*., 2004; Kepp *et al*., 2011).

Finally, although the intracellular protein profiles of MRSA isolates were obtained using the 2-DE experiments done in the course of this study, the identities of the protein spots obtained could not be ascertained because of the cost associated with further analysis (sequencing of the spots).

7.3. Recommendations for future work

Carrying out this research on a larger scale i.e. with a larger number of both UK and Nigerian MRSA isolates obtained from wider geographical locations, will go a long way to improve precision of and confidence in the findings.

Staphylococcus aureus expresses much more virulence factors than were checked for in this study. As findings from this study were not enough to determine conclusively which virulence factors were responsible for the observed differences in the virulence potential of the UK and Nigerian MRSA isolates, checking for the presence of virulence factors not checked in this study may provide a better insight to the results obtained. For instance, the presence of genes that code for cell wall anchored proteins such as the iron-regulated surface adhesins (*IsdA*, *IsdB* and *IsdH*), *S. aureus* proteins B, C, D and H, as well as those that code for secreted proteins such as the extracellular matrix protein, ESAT-6 like factor A, ESAT-6 secretion system C, etc.

Virulence is always measured relative to a standard, such as another microbe or host (Casadevall 2017). Furthermore, survival mechanisms involved in *S. aureus* intracellular persistence (whether in a vacuole or in the cytoplasm), as well as its intracellular trafficking (colocalisation with endosomal/ fluid markers, phagosomal escape and pH conditions) are highly dependent on cell origin (human or murine) and sub-type (phagocytic and non-phagocytic) as well as bacterial strain

used (Lacoma *et al*., 2017). Thus, determination of the virulence potential of the isolates used in this study with cell lines of different origins (other than the non-professional phagocytes, i.e. KB and A549 cell lines) may further shed more light on the virulence potential of the MRSA isolates.

A deeper knowledge of the biofilms produced by the MRSA isolates involved in this study will provide a better insight on the roles the biofilm plays in infection. Thus, quantification and characterisation of components of the ECM of each MRSA biofilm, as well as identification of secreted proteins specific to the MRSA biofilm mode of growth (i.e. soluble microbial products released inside the biofilms), will shed more light on the relationship between the MRSA biofilms and different aspects of infection. Furthermore, as the technique used to determine the capacity of the isolates for biofilm formation measured only a single endpoint i.e. biofilm biomass formed after fixed time periods (6 and 24 hrs), it would be useful to assess the initial steps of biofilm formation and also monitor biofilm formation for each isolate in real time.

To overcome the shortcoming associated with measuring only a single endpoint parameter as indicators of cell death, it is recommended that real-time cell analytical approaches that measure cell viability at multiple time points be used. Fluorescence based real-time measurement of cytotoxicity using non-invasive fluorescent probes (using Impermeant, DNA-Binding Dye Fluorescence) capable of long-term monitoring of cell death in real-time is recommended. Furthermore, the use of equipment capable of capturing images at regular intervals, such as the IncuCyte FLR microscope, phase contrast microscope, confocal microscope, fluorescent microscope, and quantitative phase contrast microscopy and the xCELLigence instrument is recommended. Culture based techniques such as the RealTime-Glo™ MT Cell Viability Assay (Promega) (bioluminescent assay that measures cell viability in real time to determine onset of toxicity) could also be used.

To overcome bias associated with the use of cancer cell lines, multiple cell death-relevant parameters must be measured for the precise quantification of the extent of death and its precise subroutine (Kepp *et al*., 2011). This can be done in multiplex assays which combine different chemically and biologically compatible methods to measure multiple indicators of cell death in a single sample. Some commercially available assays include the MultiTox-Fluor Multiplex Cytotoxicity Assay (Promega), MultiTox-Glo Multiplex Cytotoxicity Assay (Promega), ApoLive-Glo™ Multiplex Assay (Promega). Multiplex assays like that developed by researchers such as Gilbert *et al*., 2011, can also be used to measure multiple indicators of cell death.

While a genome remains unchanged to a large extent, the proteins in any particular cell change dramatically as genes are turned on or off in response to the environment (Cho, 2007). Therefore, comparison of the protein profiles of the MRSA isolates following exposure to different environmental conditions, such as exposure to antibiotics and growth under anaerobic conditions, may also help shed more light on any differences that exist in the virulence potentials of UK and Nigerian MRSA.

Results from this study showed that the iron-chelators studied were effective against MRSA. However, due to the high/significant toxicity of the iron-chelators on mammalian cells (KB cell line) and the contribution of DMSO (a constituent of the solvents) to this toxicity, it is recommended that work be done to modify the iChs in a bid to drastically reduce or if possible eliminate toxicity on mammalian cells after which their inhibitory effect on MRSA will be re-evaluated. Further study is also needed to elucidate the actual mode of action of the iron-chelators.

In conclusion, further work along the lines described should further clarify the basis of the enhanced virulence of Nigerian MRSA isolates and aid the development of novel strategies for combatting this important pathogen.

References

Abdulgader, S. M., Shittu, A. O., Nicol, M. P. and Kaba, M. (2015) 'Molecular epidemiology of methicillin-resistant *Staphylococcus aureus* in Africa: a systematic review', *Frontiers in Microbiology*, 6, 348

Abdullah, A. S., Moffat, C. S., Lopez-Ruiz, F. J., Gibberd, M. R., Hamblin, J. and Zerihun, A. (2017) 'Host-Multi-Pathogen Warfare: Pathogen Interactions in Co-infected Plants', *Frontiers in Plant Science*, 8, 1806

Abubakar, U. and Sulaiman, S. A. S. (2018) 'Prevalence, trend and antimicrobial susceptibility of Methicillin Resistant *Staphylococcus aureus* in Nigeria: a systematic review', *Journal of Infection and Public Health*, 11, 763-770

Adejuyigbe, E. A., Adeodu, O. O., Ako-Nai, K. A., Taiwo, O. and Owa, J. A. (2001) 'Septicaemia in high risk neonates at a teaching hospital in Ile-Ife, Nigeria', *East African Medical Journal*, 78, 540–543

Adesida, S., Abioye, O. A., Bamiro, B. S., Brai, B. I. C., Smith, S. I., Amisu, K. O., Ehichioya, D. U., Ogunsola, F. T. and Coker, A. O. (2007) 'Associated risk factors and pulsed field gel electrophoresis of nasal isolates of *Staphylococcus aureus* from medical students in a tertiary hospital in Lagos, Nigeria', *The Brazilian Journal of Infectious Diseases*, 11, 63-69

Adetayo, T. O., Deji-Agboola, A. M., Popoola, M. Y., Atoyebi, T. J and Egberongbe, K. J. (2014) 'Prevalence of methicillin resistant *Staphylococcus aureus* from clinical specimens In Ibadan, Nigeria', *The International Journal of Engineering And Science (IJES)*, 3, 1-11

Adzitey, F., Huda, N. and Ali, G. R. (2012) 'Molecular techniques for detecting and typing of bacteria, advantages and application to foodborne pathogens isolated from ducks', *3 Biotech*, 3, 97–107

Aebersold, R. and Mann, M. (2016) 'Mass-spectrometric exploration of proteome structure and function', *Nature*, 537, 347-355

Afolabi, O. A. (2008) 'Factors influencing the pattern of self-medication in an adult Nigerian population', *Anals of African Medicine*, 7, 120- 127

Agerer, F., Michel, A., Ohlsen, K. and Hauck, C. R. (2003) 'Integrin mediated invasion of *Staphylococcus aureus* into human cells requires Src family protein-tyrosine kinases', *Journal of Biological Chemistry*, 278, 42524–42531

Aggarwal, A. (2010) *Medical Microbiology Trends and Innovations*, 1st Edn., International Scientific Publishing Academy, New Delhi

Aggarwal, B. B., Gupta, S. C. and Kim, J. H. (2012) 'Historical perspectives on tumor necrosis factor and its superfamily: 25 years later, a golden journey', *Blood*, 119, 651–665

Agha M. (2012) 'Epidemiology and pathogenesis of *Clostridium difficile* and MRSA in the light of current NHS control policies: a policy review', *Annals of Medicine and Surgery*, 1, 39–43

Aizawa, K. (1971) 'Strain improvement and preservation of virulence of pathogens', In: Burges, H.D., Hussey, N.W. (eds.), *Microbial Control of Insects and Mites*, Academic Press, London, pp. 655– 672

Ajibola, O., Omisakin, O. A., Eze, A. A. and Omoleke, S. A. (2018) 'Self-Medication with antibiotics, attitude and knowledge of antibiotic aesistance among community residents and undergraduate students in Northwest Nigeria', *Diseases (Basel, Switzerland)*, 6, 32

Akagi, J., Kordon, M., Zhao, H., Matuszek, A., Dobrucki, J., Errington, R., Smith, P. J., Takeda, K., Darzynkiewicz, Z. and Wlodkowic, D. (2012) 'Real-time cell viability assays using a new anthracycline derivative DRAQ7®', *Cytometry. Part A: The Journal of the International Society for Analytical Cytology*, 83, 227–234

Akinyandenu, O. and Akinyandenu, A. (2014) 'Irrational use and non-prescription sale of antibiotics in Nigeria: A need for change', *Journal of Scientific and Innovative Research*, 3, 251-257

Ako-Nai, A. K., Lamikanra, A. B. and Onipede, A. O. (1995) 'Incidence of pathogenic microorganisms in clinical specimens from hospitals in south-western Nigeria', *East African Medical Journal*, 72, 436–441

Ako-Nai, A. K., Oluga, F. A., Onipede, A. O., Adejuyigbe, E. A. and Amusa, Y. B. (2002) 'The characterization of bacterial isolates from acute otitis media in Ile-Ife, southwestern Nigeria', *Journal of Tropical Pediatrics*, 48, 15–23

Ako-Nai, A. K., Taiwo, O., Ebri, A. and Adeniran, M. O. (1990) 'Bacterial isolates involved in cases of septicaemia in a Nigerian hospital', *East African Medical Journal*, 67, 407–412

Alavi, S. M., Sharifi, M. and Eghtesad, M. (2014) 'Bacterial sepsis: challenges of diagnosis and treatment in a teaching hospital Southwest of Iran', *Jundishapur Journal of Microbiology*, 7, e9082

Albrecht, V. S., Limbago, B. M., Moran, G. J., Krishnadasan, A., Gorwitz, R. J., McDougal, L. K., Talan, D. A., Emergency ID NET Study Group (2015) '*Staphylococcus aureus* colonization and strain type at various body sites among patients with a closed abscess and uninfected controls at U.S. emergency departments', *Journal of Clinical Microbiology*, 53, 3478–3484.

Alexander, A., Vishwanath, S., Sellvaraj, A., Varma, M., Saravu, K. and Chawla, K. (2017) 'Methicillin-resistant *Staphylococcus aureus* nasal colonisation in human immunodeficiency virus-infected patients', *Annals of Tropical Medicine and Public Health*, 10, 1809-1813

Alexander, A., Vishwanath, S., Sellvaraj, A., Varma, M., Saravu, K. and Chawla, K. (2017) 'Methicillin-resistant *Staphylococcus aureus* nasal colonization in human immunodeficiency virus-infected patients', *Annals of Tropical Medicine and Public* Health, 10, 1809-1813

Alexander, E. H., Bento, J. L., Hughes, F. M. Jr., Marriott, I., Hudson, M. C. and Bost, K. L. (2001) '*Staphylococcus aureus* and *Salmonella enterica* serovar Dublin induce tumor necrosis factor-related apoptosis-inducing ligand expression by normal mouse and human osteoblasts', *Infection and Immunity*, 69, 1581–1586

Alexander, E. H., Rivera, F. A., Marriott, I., Anguita, J., Bost, K. L. and Hudson, M. C. (2003) '*Staphylococcus aureus* - induced tumor necrosis factor - related apoptosis - inducing ligand expression mediates apoptosis and caspase-8 activation in infected osteoblasts', *BMC Microbiology*, 3(5)

Alhaji, N. B, and Isola, T. O. (2018) 'Antimicrobial usage by pastoralists in food animals in North-central Nigeria: The associated socio-cultural drivers for antimicrobials misuse and public health implications', *One Health*, 6, 41-47

Ali, R., Al-Ackhar, K., Al-Mariri, A. and Safi, M. (2014) 'Role of polymerase chain reaction in the detection of antibiotic-resistant *Staphylococcus aureus'*, *Egyptian Journal of Medical Human Genetics*, 15, 293-298

Alkharsah, K. R., Rehman, S., Alnimr, A., Diab, A., Hawwari, A. and Tokajian, S. (2018) 'Molecular typing of MRSA isolates by spa and PFGE', *Journal of King Saud University – Science*, xxx–xxx

Allen, M., Millett, P., Dawes, E. and Rushton, N. (1994) 'Lactate dehydrogenase activity as a rapid and sensitive test for the quantification of cell numbers in vitro', *Clinical Materials*, 16, 189-194

Alm, R. A., McLaughlinm R. E., Kos, V. N., Sader, H. S., Iaconis, J. P. and Lahiri, S. D. (2014) 'Analysis of *Staphylococcus aureus* clinical isolates with reduced susceptibility to ceftaroline: an epidemiological and structural perspective', J. Antimicrob. Chemother', 69, 2065–2075

Almeida, R. A., Matthews, K. R., Cifrian, E., Guidry, A. J. and Oliver, S. P. (1996) '*Staphylococcus aureus* invasion of bovine mammary epithelial cells', *Journal of Dairy Science*, 79, 1021-1026

Alsam, S., Jeong, S.R., Sissons, J., Dudley, R., Kim, K.S. and Khan, N.A. (2006) '*Escherichia coli* interactions with *Acanthamoeba*: a symbiosis with environmental and clinical implications', *Journal of Medical Microbiology*, 55, 689–694

Alva-Murillo, N., López-Meza, J. E. and Ochoa-Zarzosa, A. (2014) 'Nonprofessional phagocytic cell receptors involved in *Staphylococcus aureus* internalization', *BioMed Research International*, 2014, doi.org/10.1155/2014/538546

Alvarez, M. and Casadevall, A. (2006) 'Phagosome extrusion and host-cell survival after *Cryptococcus neoformans* phagocytosis by macrophages', *Current Biology*, 16, 2161–2165

Al-Zoubi, M. S., Al-Tayyar, I. A., Hussein, E., Al-Jabali, A. and Khudairat, S. (2015) 'Antimicrobial susceptibility pattern of *Staphylococcus aureus* isolated from clinical specimens in Northern area of Jordan', *Iranian Journal of Microbiology*, 57, 265-272

Amaral, M. M., Coelho, L. R., Flores, R. P., Souza, R. R., Silva-Carvalho, M. C., Teixeira, L. A., Ferreira-Carvalho, B. T. and Figueiredo, A. M. (2005) 'The predominant variant of the Brazilian epidemic clonal complex of methicillin-resistant *Staphylococcus aureus* has an enhanced ability to produce biofilm and to adhere to and invade airway epithelial cells', *Journal of Infectious Diseases*, 192, 801–810

Amato, S. M., Fazen, C. H., Henry, T. C., Mok, W. W., Orman, M. A., Sandvik, E. L., Volzing, K. G. and Brynildsen, M. P. (2014) 'The role of metabolism in bacterial persistence', *Frontiers in Microbiology*, 5, 70

Ambe, J. P., Gasi, I. S., Mava, Y. (2007) 'Review of neonatal infections in University of Maiduguri Teaching Hospital: common bacterial pathogens seen', *Nigerian Journal of Clinical Practice*, 10, 290-293

Andrews, J. M. (2006) 'Determination of minimum inhibitory concentrations', Department of Microbiology, City Hospital NHS Trust, Birmingham, B18 7QH

Andrews, S. C., Robinsin, A. K. and Rodriguez-Quinones, F. (2003) 'Bacterial iron homeostasis', *FEMS Microbiology Reviews*, 27, 215-237

Anguzu, J. R. and Olila, D. (2007) 'Drug sensitivity patterns of bacterial isolates from septic post-operative wounds in a regional referral hospital in Uganda', *African Health Sciences*, 7, 148-154

Archer, G. L. (1998) '*Staphylococcus aureus*: a well-armed pathogen', *Clinical Infectious Diseases*, 26, 1179-1181

Archer, G. L. and Niemeyer, D. M. (1994) 'Origin and evolution of DNA associated with resistance to methicillin in staphylococci', *Trends in Microbiology*, 2, 343–347

Arciola, C. R., Campoccia, D., Ravaioli, S. and Montanaro, L. (2015) 'Polysaccharide intercellular adhesin in biofilm: structural and regulatory aspects', *Frontiers in Cellular and Infection Microbiology*, 5, 7

Ashkenazi, A., et al. (1998) 'Death receptors: signaling and modulation', *Science*, 281, 1305–1308

Ashraf, S., Cheng, J. and Zhao, X. (2017) 'Clumping factor A of *Staphylococcus aureus* interacts with AnnexinA2 on mammary epithelial cells', *Scientific Reports*, 7, 40608

Aslam, B., Basit, M., Nisar, M. A., Khurshid, M. and Rasool, M. H. (2017) 'Proteomics: technologies and their applications', *Journal of Chromatographic Science*, 55, 182–196

Atshan, S. S., Shamsudin, M. N., Sekawi, Z., Thian Lung, L. T., Barantalab, F., Liew, Y. K., Alreshidi, M. A., Abduljaleel, S. A. and Hamat, R. A. (2015) 'Comparative proteomic

analysis of extracellular proteins expressed by various clonal types of *Staphylococcus aureus* and during planktonic growth and biofilm development', *Frontiers in Microbiology*, 6, 524

Awosan, K. J., Ibitoye, P. K. and Abubakar, A. K. (2018) Knowledge, risk perception and practices related to antibiotic resistance among patent medicine vendors in Sokoto metropolis, Nigeria', *Nigerian Journal of Clinical Practice*, 21, 1476-1483

Ayukekbong, J. A., Ntemgwa, M. and Atabe, A. N. (2017) 'The threat of antimicrobial resistance in developing countries: causes and control strategies', *Antimicrobial Resistance and Infection Control*, 6, 47

Azeredo, J., Azevedo, N. F., Briandet, R., Cerca, N., Coenye, T., Costa, A. R., Desvaux, M., Di Bonaventura, G., Hébraud, M., Jaglic, Z., Kačániová, M., Knøchel, S., Lourenço, A., Mergulhão, F., Meyer, R. L., Nychas, G., Simões, M., Tresse, O. and Sternberg, C. (2017) 'Critical review on biofilm methods', *Critical Reviews In Microbiology*, 43, 313–351

Baba, T., Kuwahara-Arai, K., Uchiyama, I., Takeuchi, F., Ito, T. and Hiramatsu K. (2009) 'Complete genome sequence of *Macrococcus caseolyticus* strain JCSCS5402, [corrected] reflecting the ancestral genome of the human-pathogenic staphylococci', *Journal of Bacteriology*, 191, 1180–1190

Babinchak, T., Ellis-Grosse, E., Dartois, N., Rose, G. M. and Loh. E. Tigecycline 301 and 306 Study Groups (2005) 'The efficacy and safety of tigecycline for the treatment of complicated intra-abdominal infections: analysis of pooled clinical trial data', *Clinical Infectious Diseases*, 41(Supplement 5), S354-S367

Backert, S. and Hofreuter, D. (2013) 'Molecular methods to investigate adhesion, transmigration, invasion and intracellular survival of the foodborne pathogen *Campylobacter jejuni*', *Journal of Microbiological Methods*, 95, 8–23

Baig, S., Johannesen, T. B., Overballe-Petersen, S., Larsen, J., Larsen, A. R., and Stegger, M. (2018) 'Novel SCC mec type XIII (9A) identified in an ST152 methicillin-resistant *Staphylococcus aureus*' *Infection, Genetics and Evolution*, 61, 74–76

Baines, S. L., Holt, K. E., Schultz, M. B., Seemann, T., Howden, B. O., Jensen, S. O., van Hal, S. J., Coombs, G. W., Firth, N., Powell, D. R., Stinear, T. P. and Howden, B. P. (2015) 'Convergent adaptation in the dominant global hospital clone ST239 of methicillin-resistant *Staphylococcus aureus', mBio*, 6, e00080-15

Balasubramanian, D., Harper, L., Shopsin, B. and Torres, V. J. (2017) '*Staphylococcus aureus* pathogenesis in diverse host environments', *Pathogens and Disease*, 75, ftx005

Balwit, J. M., van Langevelde, P., Vann, J. M. and Proctor, R. A. (1994) 'Gentamicin-resistant menadione and hemin auxotrophic *Staphylococcus aureus* persist within cultured endothelial cells', *Journal of Infectious Diseases*, 170, 1033–1037

Bantel, H., Sinha, B., Domschke, W., Peters, G., Schulze-Osthoff, K. and Janicke, R. U. (2001) 'Toxin is a mediator of *Staphylococcus aureus*-induced cell death and activates caspases via the intrinsic death pathway independently of death receptor signaling', *Journal of Cell Biology*, 155, 637–648

Basch, H. and Gadebusch H. H. (1968) '*In vitro* antimicrobial activity of dimethylsulfoxide', *Applied Microbiology*, 16(12)

Bassetti, M., Peghin, M., Trecarichi, E. M., Carnelutti, A., Righi, E., Del Giacomo, P., Ansaldi, F., Trucchi, C., Alicino, C., Cauda, R., Sartor, A., Spanu, T., Scarparo, C. and Tumbarello, M. (2017) 'Characteristics of *Staphylococcus aureus* bacteraemia and predictors of early and late mortality', *PLoS One*, 12:e0170236

Bassetti, M., Trecarichi, E. M., Mesini, A., Spanu, T., Giacobbe, D. R., Rossi, M., Shenone, E., Pascale, G. D., Molinari, M. P., Cauda, R., Viscoli, C. and Tumbarello, M. (2011) 'Risk factors and mortality of healthcare-associated and community-acquired *Staphylococcus aureus* bacteraemia', *Clinical Microbiology Infections*, http://dx.doi.org/10.1111/j.1469-0691.2011.03679.x

Bayer, A. S., Schneider, T. and Sahl, H. G. (2013) 'Mechanisms of daptomycin resistance in *Staphylococcus aureus*: role of the cell membrane and cell wall', *Annals of the New York Academy of Sciences*, 1277, 139–158

Bayles, K. W., Wesson, C. A., Liou, L. E., Fox, L. K., Bohach, G. A. and Trumble, W. R. (1998) 'Intracellular *Staphylococcus aureus* escapes the endosome and induces apoptosis in epithelial cells', *Infection and Immunity*, 66, 336–342

Becker, A. P., Cantareli, V. V., Rossato, F. C. P., Inoue, F. M., Dias, C. and d'Azevedo, P. A. (2014) 'Non-multidrug-resistant, Methicillin-Resistant *Staphylococcus aureus* causing infection in health-care facilities in southern Brazil', *Journal of Medical Microbiology and Diagnosis*, 3:3

Becker, K., Friedrich, A. W., Lubritz, G., Weilert, M., Peters, G. and Von Eiff, C. (2003) 'Prevalence of genes encoding pyrogenic toxin superantigens and exfoliative toxins among strains of *Staphylococcus aureus* isolated from blood and nasal specimen', *Journal of Clinical Microbiology*, 41, 1434–1439

Beilouny, B., Ciupea, A., Eloy, C.and Simon, G. (2008) 'Fatal community-acquired pneumonia due to *Staphylococcus aureus* carrying Panton–Valentine leukocidin genes after a stay in Africa', *Intensive Care Medicine*, 34, 388–389

Bell, J. M., and Turnidge, J. D. (2002) 'High prevalence of oxacillin-resistant *Staphylococcus aureus* isolates from hospitalized patients in Asia-Pacific and South Africa: results from SENTRY antimicrobial surveillance program, 1998–1999', *Antimicrobial Agents and Chemotherapy*, 46, 879–881.

Bens, C. P. M., Voss, A. and Klaassen, C. H. W. (2006) 'Presence of a novel DNA methylation enzyme in methicillin-resistant *Staphylococcus aureus* isolates associated with pig farming leads to uninterpretable results in standard pulsed-field gel electrophoresis analysis', *Journal of Clinical Microbiology*, 44, 1875–1876

Bera, A., Herbert, S., Jakob, A., Vollmer, W. and Gotz, F. (2005) 'Why are pathogenic staphylococci so lysozyme resistant? The peptidoglycan O-acetyltransferase OatA is the major determinant for lysozyme resistance of *Staphylococcus aureus*', *Molecular Microbiology* 55, 778–787

Berkelman, T. and Stenstedt, T. (2004) *2-D Electrophoresis Principles and Methods*, GE Healthcare Bio-Sciences AB, Uppsala, Sweden

Bien, J., Sokolova, O. and Bozko, P. (2011) 'Characterization of virulence factors of *Staphylococcus aureus*: novel function of known virulence factors that are implicated in activation of airway epithelial pro-inflammatory response', *Journal of Pathogens*, doi: 10.4061/2011/601905

Bischoff, M., Dunman, P., Kormanec, J., Macapagal, D., Murphy, E., Mounts, W., Berger-Bächi, B. and Projan, S. (2004) 'Microarray-based analysis of the *Staphylococcus aureus* σ^B regulon', *Journal of Bacteriology*, 186, 4085–4099.

Blaser, M. J. and Kirschner, D. (2007) 'The equilibria that allow bacterial persistence in human hosts', *Nature*, 449, 843-849

Bohach, G. A., Dinges, M. M., Mitchell, D. T., Ohlendorf, D. H. and Schlievert, P. M. (1997) 'Exotoxins', p. 83–111. *In* K. B. Crossley and Archer, G. L. (ed.), *The staphylococci in human disease*, Churchill Livingstone, Inc., New York, N.Y.

Bohach, G. A., Fast, D. J., Nelson, R. D. and Schlievert, P. M. (1990) 'Staphylococcal and streptococcal pyrogenic toxins involved in toxic shock syndrome and related illnesses', *Critical Reviews in Microbiology*, 17, 251–272

Bonofiglio, L., Mollerach, M. and Gardella, N. (2012) 'Application of molecular typing methods to the study of medically relevant Gram-positive cocci', *Gel Electrophoresis–Advanced Techniques*, 113

Booth, M. C., Pence, L. M., Mahasreshti, P., Callegan, M. C. and Gilmore, M. S. (2001) 'Clonal associations among *Staphylococcus aureus* isolates from various sites of infection', *Infection and Immunity*, 69, 345–352

Boswihi, S. S. and Udo, E. E. (2018) 'Methicillin-resistant *Staphylococcus aureus*: An update on the epidemiology, treatment options and infection control', *Current Medicine Research and Practice*, 8, 18–24

Boswihi, S. S., Udo, E. E. and Al-Sweih, N. (2016) 'Shifts in the Clonal Distribution of Methicillin-Resistant *Staphylococcus aureus* in Kuwait Hospitals: 1992-2010', *PLoS One*, 11, e0162744

Boucher, H. W. and Corey, G. R. (2008) 'Epidemiology of methicillin-resistant *Staphylococcus aureus*', *Clinical Infectious Diseases*, 46, S344–S349

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B., Scheld, M., Spellberg, B. and Bartlett, J. (2009) 'Bad bugs, no Drugs: no ESKAPE! An update from the infectious diseases society of America', *Clinical Infectious Diseases*, 48, 1–12

Bouchiat, C., Moreau, K., Devillard, S., Rasigade, J-P., Mosnier, A., Geissmann, T., Bes, M., Tristan, A., Lina, G., Laurent. F., Piroth, L., Aissa, N., Duval, X., Le Moing, V., Vandenesch, F., the French VIRSTA Study Group (2015) '*Staphylococcus aureus* infective endocarditis versus bacteremia strains: Subtle genetic differences at stake', *Infection, Genetics and Evolution*, 36, 524–530

Boyle-Vavra, S., Berke, S. K., Lee, J. C. and Daum, R. S. (2000) 'Reversion of the glycopeptide resistance phenotype in *Staphylococcus aureus* clinical isolates', *Antimicrobial Agents and Chemotherapy*, 44, 272–277

Bozcal, E. and Dagdeviren, M. (2017) 'Toxicity of β-Lactam antibiotics: pathophysiology, molecular biology and possible recovery strategies. In Ntambwe Malangu, Poisoning IntechOpen, DOI: 10.5772/intechopen.70199, pages 87-105

Brandis, G., Cao, S., Huseby, D. L. and Hughes, D. (2017) 'Having your cake and eating it - *Staphylococcus aureus* small colony variants can evolve faster growth rate without losing their antibiotic resistance', *Microbial Cell (Graz, Austria)*, 4, 275–277

Breurec, S., Fall, C., Pouillot, R., Boisier, P., Brisse, S., Diene-Sarr, F., Djibo, S., Etienne, J., Fonkoua, M. C., Perrier-Gros-Claude, J. D., Ramarokoto, C. E., Randrianirina, F., Thiberge, J. M. and Zriouil, S. B.; Working Group on *Staphylococcus aureus* Infections, Garin B, Laurent F. (2011) 'Epidemiology of methicillin-susceptible *Staphylococcus aureus* lineages in five major African towns: high prevalence of Panton-Valentine leukocidin genes', *Clinical Microbiology and Infection*, 17, 633-639

Brouillette, E., Talbot, B. G. and Malouin, F. (2003) 'The fibronectin-binding proteins of *Staphylococcus aureus* may promote mammary gland colonization in a lactating mouse model of mastitis', Infection and Immunity, 71, 2292-2295

Brown, A. F., Leech, J. M., Rogers, T. R. and McLoughlin, R. M. (2014) '*Staphylococcus aureus* colonization: modulation of host immune response and impact on human vaccine design', *Frontiers in Immunology*, 4, 507

Brown, M. and Wittwer C. (2000) 'Flow cytometry: Principles and clinical applications in hematology', *Clinical Chemistry*, 46, 1221–1229

Brown, N. F., Wickham, M. E., Coombes, B. K. and Finlay, B. B. (2006) 'Crossing the line: selection and evolution of virulence traits', *PLOS Pathogens*, 2, e42

Brown, N. M., Goodman, A., Horner, C., Jenkins, A. and Brown, E. (2018) Guidelines for the prophylaxis and treatment Of MRSA in the UK: 2018 update', *British Society for Antimicrobial Chemotherapy*, www.his.org.uk/media

Bubeck, W, J., Williams, W. A. and Missiakas, D. (2006) 'Host defenses against *Staphylococcus aureus* infection require recognition of bacterial lipoproteins', *Proceedings of the National Academy of Sciences of the United States of America*, 103, 13831-13836

Bur, S., Preissner, K. T., Herrmann, M. and Bischoff, M. (2013) 'The *Staphylococcus aureus* extracellular adherence protein promotes bacterial internalization by keratinocytes independent of fibronectin-binding proteins', *Journal of Investigative Dermatology*, 133, 2004-2012

Burdette, S. D., Watkins, R. R., Wong, K. K., Mathew, S. D., Martin, D. J. and Markert, R. J. (2012). *Staphylococcus aureus* pyomyositis compared with non-*Staphylococcus aureus* pyomyositis', *Journal of Infection*, 64, 507–512

Burke, F. M., McCormack, N., Rindi, S., Speziale, P. and Foster, T. J. (2010) 'Fibronectin-binding protein B variation in *Staphylococcus aureus*', *BMC Microbiology*, 10, 160

Buss, J. L., Greene, B. T., Turner, J., Torti, F. M. and Torti, S. V. (2004) 'Iron chelators in cancer chemotherapy', *Current Topics in Medicinal Chemistry*, 4, 1623–1635

Buss, J. L., Torti, F. M. and Torti, S. V. (2003) 'The role of iron chelation in cancer therapy', *Current Medicinal Chemistry*, 10, 1021–1034

Butte, A. (2002) 'The use and analysis of microarray data', *Nature Reviews Drug Discovery*, 1, 51–60

Calderón, C. B. and Sabundayo, P. B. (2007) Antimicrobial Classifications: Drugs for Bugs. In: Schwalbe, R., Steele-Moore, L. and Goodwin, A. C. *Antimicrobial Susceptibility Testing Protocols*. Boca Raton: Taylor & Francis Group. 7-47

Cameron, D. R., Howden, B. P. and Peleg, A. Y. (2011) 'The interface between antibiotic resistance and virulence in *Staphylococcus aureus* and its impact upon clinical outcomes', *Clinical Infectious Diseases*, 53, 576–582

Cardas, M., Khan, N.A. and Alsam, S. (2012) '*Staphylococcus aureus* exhibit similarities in their interactions with Acanthamoeba and ThP1 macrophage-like cells', *Experimental Parasitology*, 132, 513–518

Casadevall, A. (2017) 'The pathogenic potential of a microbe', *mSphere*, 2, e00015-17

Casadevall, A. and Pirofski, L. (2001) 'Host-pathogen interactions: the attributes of virulence', *Journal of Infectious Diseases*, 184, 337-344

Casadevall, A. and Pirofski, L. (2009) 'Virulence factors and their mechanisms of action: the view from a damage–response framework', *Journal of Water and Health,* 7, Suppl 1: S2-S18

Casadevall, A. and Pirofski, L. A. (2000) 'Host-pathogen interactions: basic concepts of microbial commensalism, colonization, infection, and disease. Infection and immunity', 68, 6511–6518

Cassat, J. E. and Skaar, E. P. (2013) 'Iron in infection and immunity', *Cell Host and Microbe,* 13, 509–519

Castro-Escarpulli, G., Aguilar, N. M. A., Sánchez, G. R., Bocanegra-Garcia, V., Guo, X., Juárez-Enríquez, S. R., Luna-Herrera, J., Martínez, C. M. and Guadalupe, A. G. M. (2015) 'Identification and typing methods for the study of bacterial infections: a brief review and mycobacterial as case of study'*Archives of Clinical Microbiology*, 7:1

CDC - Centers for Disease Control and Prevention (2011) 'Vital signs: central line-associated blood stream infections—United States, 2001, 2008, and 2009', *Morbidity and Mortality Weekly Report,* 60, 243–248

CDC- Centers for Disease Control and prevention (2013)' Antibiotic Resistance Threats in the United States, 508', Available online at: http://www.cdc.gov/ drugresistance/threat-report-2013/pdf/ar-threats-2013-508.pdf

Cellier, M. F., Courville, P. and Campion, C. (2007) 'Nramp1 phagocyte intracellular metal withdrawal defense', *Microbes and Infection*, 9, 1662–1670

Ceri, H., Olson, M. E., Stremick, C., Read, R. R., Morck, D. and Buret, A. (1999) 'The Calgary biofilm device: new technology for rapid determination of antibiotic susceptibilities of bacterial biofilms', *Journal of Clinical Microbiology,* 37, 1771–1776

Chambers, H. F. (1997) 'Methicillin resistance in staphylococci: molecular and biochemical basis and clinical implications', *Clinical Microbiology Reviews*, 10, 781–791

Chambers, H. F. (2001) 'The changing epidemiology of *Staphylococcus aureus*?', *Emerging Infectious Diseases*', 7, 178–182

Chan, F. K., Moriwaki, K. and De Rosa, M. J. (2013) 'Detection of necrosis by release of lactate dehydrogenase activity', *Methods in Molecular Biology*, 979, 65–70

Chan, G. C., Chan, S., Ho, P. L. and Ha, S. Y. (2009) 'Effects of chelators (deferoxamine, deferiprone and deferasirox) on the growth of *Klebsiella pneumonia* and *Aeromonas hydrophila* isolated from transfusion-dependent thalassemia patients', *Hemoglobin,* 33, 352–360

Chan, P. F., and Foster, S. J. (1998) 'Role of *SarA* in virulence determinant production and environmental signal transduction in *Staphylococcus aureus*', *Journal of Bacteriology,* 180, 6232-6241

Chan, Y. G. Y., Kim, H. K., Schneewind, O., and Missiakas, D. (2014) 'The capsular polysaccharide of *Staphylococcus aureus* is attached to peptidoglycan by the LytR-CpsA-Psr (LCP) family of enzymes', *The Journal of Biological Chemistry*, 289, 15680–15690

Chang, J., Ye, D., Lv, B., Jiang, M., Zhu, S., Yan, K., Tian, Y. and Fang, Y. (2017) 'Sale of antibiotics without a prescription at community pharmacies in urban China: a multicentre cross-sectional survey', *Journal of Antimicrobial Chemotherapy*, 72, 1235-1242

Changchien, C. H., Chen, Y. Y., Chen, S. W., Chen, W. L., Tsay, J. G. and Chu, C. (2011) 'Retrospective study of necrotizing fasciitis and characterization of its associated methicillin-resistant *Staphylococcus aureus* in Taiwan', *BMC Infectious Diseases*, 11(297)

Chapaval, L., Moon, D. H., Gomes, J. E., Duarte, F. R. and Tsai, S. M. (2006) 'Use of pcr to detect classical enterotoxins genes (ent) and toxic shock syndrome toxin-1 gene (tst) in *Staphylococcus aureus* isolated from crude milk and determination of toxin productivities of S. aureus isolates harboring these genes', *Arquivos do Instituto Biológico, São Paulo*, 73, 165-169

Chastre, J., Blasi, F., Masterton, R. G., Rello, J., Torres, A. and Welte, T. (2014) 'European perspective and update on the management of nosocomial pneumonia due to methicillin-resistant *Staphylococcus aureus* after more than 10 years of experience with linezolid', *Clinical Microbiology and Infection*, Suppl 4, 19-36

Chatterjee, S. S. and Otto, M. (2013) 'Improved understanding of factors driving methicillin-resistant *Staphylococcus aureus* epidemic waves', *Clinical Epidemiology*, 5, 205-217

Chaturvedi, A., Garg, R. and Singh, V. A. (2017) 'Comparative evaluation of different culture media for the isolation and identification of common urinary pathogens', *International Journal of Research in Medical Sciences*', 5, 3676-3679

Chaudhary, R., Thapa, S. K., Rana, J. C., Shah, P. K. (2017) 'Surgical site infections and antimicrobial resistance pattern', *Journal of Nepal Health Research Council*, 15, 120-123

Chaudhry, S. B., Veve, M. P. and Wagner, J. L. (2019) 'Cephalosporins: a focus on side chains and β-lactam cross-reactivity', *Pharmacy*, 7, 103

Chauhan, A. K. and Varma, A. A (2009) '*Textbook of Molecular Biotechnology'*, I.K. International Publishing House Pvt. Ltd., New Delhi, India

Chaytor, J. L., Tokarew, J. M., Wu, L. K., Leclère, M., Tam, R. Y., Capicciotti, C. J., Guolla, L., von Moos, E., Findlay, C. S., Allan, D. S. and Ben, R. N. (2012) 'Inhibiting ice recrystallization and optimization of cell viability after cryopreservation', *Glycobiology*, 22, 123-133

Chen, C. J. and Huang, Y. C. (2018) 'Emergence of livestock-associated methicillin-resistant *Staphylococcus aureus*: Should it be a concern?', *Journal of the Formosan Medical Association*, pii: S0929-6646(18)30114-1.

Chen, X. P., Li, W. G., Zheng, H., Du, H. Y., Zhang, L., Zhang, L., Che, J., Wu, Y., Liu, S. M. and Lu, J. X. (2017) 'Extreme diversity and multiple SCC*mec* elements in coagulase-negative Staphylococcus found in the clinic and community in Beijing, China', *Annals of Clinical Microbiology and Antimicrobials*, 16, 57

Chen, X., Wang, W. K., Han, L. Z., Liu, Y., Zhang, H., Tang, J., Liu, Q. Z., Huangfu, Y. C. and Ni, Y. X. (2013) 'Epidemiological and genetic diversity of staphylococcus aureus causing bloodstream infection in Shanghai, 2009-2011', *PLoS ONE*, 8, e72811.

Cheung, A. I., Projan, S. J., Edelstein, R. E. and Fischetti, V. A. (1995) 'Cloning, expression, and nucleotide sequence of a *Staphylococcus aureus* gene (fbpA) encoding a fibrinogen-binding protein', *Infection and Immunity*, 63, 1914-1920

Cheung, A. L., Nishina, K. A., Trotonda, M. P., and Tamber, S. (2007) 'The SarA protein family of *Staphylococcus aureus*', *The International Journal of Biochemistry and Cell Biology*, 40, 355–361

Cheung, A. L., Bayer, A. S., Zhang, G., Gresham, H.' and Xiong, Y. Q. (2004) 'Regulation of virulence determinants *in vitro* and *in vivo* in *Staphylococcus aureus*', *FEMS Immunology and Medical Microbiology*, 15, 1-9

Cheung, G. Y., Wang, R., Khan, B. A., Sturdevant, D. E. and Otto, M. (2011) 'Role of the accessory gene regulator *agr* in community-associated methicillin-resistant *Staphylococcus aureus* pathogenesis', *Infection and Immunity*, 79, 1927–1935

Chiaraviglio, L. and Kirby, J. E. (2014) 'Evaluation of impermeant, DNA-binding dye fluorescence as a real-time readout of eukaryotic cell toxicity in a high throughput screening format', *Assay and Drug Development Technologies*, 12, 219–228

Cho, W. C. (2007) 'Proteomics technologies and challenges', *Genomics, Proteomics and Bioinformatics*, 5, 77–85

Chongtrakool, P., Ito, T., Ma, X. X., Kondo, Y., Trakulsomboon, S., Tiensasitorn, C., Jamklang, M., Chavalit, T., Song, J. H. and Hiramatsu, K. (2006) 'Staphylococcal cassette chromosome *mec* (*SCCmec*) typing of methicillin-resistant *Staphylococcus aureus* strains isolated in 11 Asian countries: a proposal for a new nomenclature for *SCCmec* elements', *Antimicrobial Agents and Chemotherapy*, 50, 1001–1012

Choo, E. J. and Chambers, H. F. (2016) 'Treatment of Methicillin-Resistant *Staphylococcus aureus* bacteraemia', *Infection and Chemotherapy*, 48, 267-273

Chu, V. H., Crosslin, D. R., Friedman, J. Y., Reed, S. D., Cabell, C. H., Griffiths, R. I., Masselink, L. E., Kaye, K. S., Corey, G. R., Reller, L. B., Stryjewski, M. E., Schulman, K. A. and Fowler, V. G. Jr (2005) '*Staphylococcus aureus* bacteremia in patients with prosthetic devices: costs and outcomes', *American Journal of Medicine*, 118(1416)

Cilloniz, C., Martin-Loeches, I., Garcia-Vidal, C., San Jose, A., and Torres, A. (2016) 'Microbial etiology of pneumonia: epidemiology, diagnosis and resistance patterns', International journal of molecular sciences Molecular Diversity Preservation International, 17, 2120

Clinton, A. and Carter, T. (2015) 'Chronic wound biofilms: pathogenesis and potential therapies', *Lab Medicine*, 46, 277-284

Cluzet, V. C., Gerber, J. S., Nachamkin, I., Metlay, J. P., Zaoutis, T. E., Davis, M. F., Julian, K. G., Linkin, D. R., Coffin, S. E., Margolis, D. J., Hollander, J. E., Bilker, W. B., Han, X., Mistry, R. D., Gavin, L. J., Tolomeo, P., Wise, J. A., Wheeler, M. K., Hu, B., Fishman, N. O., Royer, D. and Lautenbach, E. (2015) 'Risk factors for recurrent colonization with methicillin-resistant *Staphylococcus aureus* in community-dwelling adults and children', *Infection Control and Hospital Epidemiology*, 36, 786–793

Cockfield, J. D., Pathak, S., Edgeworth, J. D. and Lindsay, J. A. (2007) 'Rapid determination of hospital-acquired meticillin-resistant *Staphylococcus aureus* lineages', *Journal of Medical Microbiology,* 56, 614-619

Coia, J. E., Leanord, A. T. and Reilly, J. S. (2014) 'Screening for meticillin resistant *Staphylococcus aureus* (MRSA): who, when, and how?', *British Medical Journal*, 348, g1697

Collins, J., Rudkin, J., Recker, M., Pozzi, C., O'Gara, J. P., and Massey, R. C. (2010) 'Offsetting virulence and antibiotic resistance costs by MRSA', *International Society for Microbial Ecology*, 10, 1751-7362

Cookson, B. D., Robinson, D. A., Monk, A. B., Murchan, S., Deplano, A., de Ryck, R., Struelens, M. J., Scheel, C., Fussing, V., Salmenlinna, S., Vuopio-Varkila, J., Cuny, C., Witte, W., Tassios, P. T., Legakis, N. J., van Leeuwen, W., van Belkum, A., Vindel, A., Garaizar, J., Haeggman, S., Olsson-Liljequist,B., Ransjo, U., Muller-Premru, M., Hryniewicz, W., Rossney, A., O'Connell, B., Short, B. D., Thomas, J., O'Hanlon, S. and Enright, M. C. (2007) 'Evaluation of molecular typing methods in characterizing a European collection of epidemic methicillin-resistant *Staphylococcus aureus* strains: the HARMONY collection', *Journal of Clinical Microbiology*, 45, 1830–1837

Corrado, A., Donato, P., Maccari, S., Cecchi, R., Spadafina, T., Arcidiacono, L., Tavarini, S., Sammicheli, C., Laera, D., Manetti, A. G. O., Ruggiero, P., Galletti, B., Nuti, S., De Gregorio, E., Bertholet, S., Seubert, A., Bagnoli, F., Bensi, G. and Chiarot, E. (2016) '*Staphylococcus aureus*-dependent septic arthritis in murine knee joints: local immune response and beneficial effects of vaccination', *Sci. Rep*, 6, 38043

Corredor Arias, L. F., Luligo Espinal, J. S., Moncayo Ortiz, J. I., Santacruz Ibarra, J. J. and Alvarez Aldana, A. (2016) 'Relationship between super antigenicity, antimicrobial resistance and origin of *Staphylococcus aureus* isolated', *Colombia Medica*, 47, 15–20

Corti, N. and Imhof, A. (2009) 'Chapter 26 - Miscellaneous antibacterial drugs', *Side Effects of Drugs Annual*, 31. 427-455

Cosgrove, S. E., Sakoulas, G., Perencevich, E. N., Schwaber, M. J., Karchmer, A. W., and Carmeli, Y. (2003). Comparison of mortality associated with methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* bacteremia: a meta-analysis', *Clinical and Infectious Diseases*, 36, 53–59

Costa, A. R., Batista, D. W. F., Ribas, R. M., Sousa, A. M., Pereira, M. O. And Botelho, C. M. (2013) 'Staphylococcus aureus virulence factors and disease', Microbial pathogens and strategies for combating them: science, technology and education, A. Méndez-Vilas, Ed., Formatex Spain

Costerton, J. W., Stewart, P. S. and Greenberg, E. P. (1999) 'Bacterial biofilms: a common cause of persistent infections', *Science*, 284, 1318-1322

Couto, I., de Lencastre, H., Severina, E., Kloos, W., Webster, J. A., Hubner R. J., Sanches, I. S. and Tomasz, A. (1996) 'Ubiquitous presence of a *mecA* homologue in natural isolates of *Staphylococcus sciuri*', *Microbial Drug Resistance*, 2, 377–391

Cox, G., Stogios, P. J., Savchenko, A., and Wright, G. D. (2015) 'Structural and molecular basis for resistance to aminoglycoside antibiotics by the Adenylyltransferase ANT (2″)-Ia', *mBio*, 6, e02180–14

Cox, J. and Mann, M. (2011) 'Quantitative, high-resolution proteomics for data-driven systems biology', *Annual Review of Biochemistry*, 80, 273-299

Craigen, M. A., Watters, J. and Hackett, J. S. (1992) 'The changing epidemiology of osteomyelitis in children', *Journal of Bone and Joint Surgery*, 74, 541-545

Crisostomo, M. I., Westh, H., Tomasz, A, Chung, M., Oliveira, D. C. and Lencastre, H. (2001) 'The evolution of methicillin resistance in *Staphylococcus aureus*: similarity of genetic backgrounds in historically early methicillin-susceptible and -resistant isolates and contemporary epidemic clones', *Proceedings of the National Academy of Sciences*, 98, 9865-9870

Curreem, S. O., Watt, R. M., Lau, S. K. and Woo, P. C. (2012) 'Two-dimensional gel electrophoresis in bacterial proteomics', *Protein and Cell*, 3, 346–363

Curry W. J. and Lewis P. R. (2003) Bacteremia and Sepsis. In: Taylor R.B., David A.K., Fields S.A., Phillips D.M., Scherger J.E. (eds) *Family Medicine*. Springer, New York, NY

Czabotar, P. E., Lessene, G., Strasser, A. and Adams, J. M. (2014) 'Control of apoptosis by the BCL-2 protein family: implications for physiology and therapy', *Nature Reviews Molecular Cell Biology*, 15, 49–63

Da Violante, G., Zerrouk, N., Richard, I., Provot, G., Chaumeil, J. C. and Arnaud P. (2002) 'Evaluation of the cytotoxicity effect of dimethyl sulfoxide (DMSO) on Caco2/TC7 colon tumor cell cultures', *Biological and Pharmaceutical Bulletin*, 25, 1600-1603

Darisipudi, M. N., Nordengrün, M., Bröker, B. M., and Péton, V. (2018) 'Messing with the Sentinels-The Interaction of *Staphylococcus aureus* with dendritic cells', *Microorganisms*, 6, 87

Das, S., Lindemann, C., Young, B. C., Muller, J., Österreich, B., Ternette, N., Winkler, A. C., Paprotka, K., Reinhardt, R., Förstner, K. U., Allen, E., Flaxman, A., Yamaguchi, Y., Rollier, C. S., van Diemen, P., Blättner, S., Remmele, C. W., Selle, M., Dittrich, M., Müller, T., Vogel, J., Ohlsen, K., Crook, D. W., Massey, R., Wilson, D. J., Rudel, T., Wyllie, D. H. and Fraunholz, M. J. (2016) 'Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation', *Proceedings of the National Academy of Sciences of the United States of America*, 113, E3101–E3110

Das, S., Lindemann, C., Young, B. C., Muller, J., Österreich, B., Ternette, N., Winkler, A. C., Paprotka, K., Reinhardt, R., Förstner, K. U., Allen, E., Flaxman, A., Yamaguchi, Y., Rollier, C. S., van Diemen, P., Blättner, S., Remmele, C. W., Selle, M., Dittrich, M., Müller, T., Vogel, J., Ohlsen, K., Crook, D. W., Massey, R., Wilson, D. J., Rudel, T., Wyllie, D. H. and Fraunholz, M. J. (2016) 'Natural mutations in a *Staphylococcus aureus* virulence regulator attenuate cytotoxicity but permit bacteremia and abscess formation', *Proceedings of the National Academy of Sciences of the United States of America*, 113, E3101-3110

Daum, R. S. (2007) 'Skin and soft-tissue infections caused by methicillin-resistant *Staphylococcus aureus*', *New England Journal of Medicine*, 357, 380–390

David, M. Z. and Daum, R. S. (2010) 'Community-associated methicillin-resistant *Staphylococcus aureus*: epidemiology and clinical consequences of an emerging epidemic', *Clinical Microbiology Reviews*, 23, 616–687

David, M. Z., Mennella, C., Mansour, M., Boyle-Vavra, S. and Daum, R. S. (2008) 'Predominance of methicillin-resistant *Staphylococcus aureus* among pathogens causing skin and soft tissue infections in a large urban jail: risk factors and recurrence rates', *Journal of Clinical Microbiology*, 46, 3222–3227

David, M. Z., Taylor, A., Lynfield, R., Boxrud, D. J., Short, G., Zychowski, D., Boyle-Vavra, S. and Daum, R. S. (2013) 'Comparing pulsed-field gel electrophoresis with multilocus sequence typing, *spa* typing, staphylococcal cassette chromosome *mec* (SCCm*ec*) typing and PCR for Panton-Valentine leukocidin, *arcA* and *opp3* in methicillin-resistant *Staphylococcus aureus* isolates at a US Medical Center', *Journal of Clinical Microbiology*, 51, 814-819

Davies, T. A., Page, M. G. P., Shang, W., Andrew, T., Kania, M. and Bush, K. (2007) 'Binding of ceftobiprole and comparators to the penicillin-binding proteins of *Escherichia coli*,

Pseudomonas aeruginosa, Staphylococcus aureus, and *Streptococcus pneumoniae*', Antimicrob. Agents Chemother, 51, 2621–2624

Day, N. P., Moore, C. E., Enright, M. C., Berendt, A. R., Smith, J. M., Murphy, M. F., Peacock, S. J., Spratt, B. G. and Feil, E. J. (2001) 'Retraction', *Science*, 295, 971

De Gorgolas, M., Aviles, P., Verdejo, C. and Fernández Guerrero, M. L. (1995) 'Treatment of experimental endocarditis due to methicillin-susceptible or methicillin-resistant *Staphylococcus aureus* with trimethoprim-sulfamethoxazole and antibiotics that inhibit cell wall synthesis', *Antimicrobial Agents and Chemotherapy*, 39, 953–957

De Haas, C.J., Veldkamp, K.E., Peschel, A., Weerkamp, F., Van Wamel, W.J., Heezius, E.C., Poppelier, M.J., Van Kessel, K.P. and Van Strijp,J.A. (2004) 'Chemotaxis inhibitory protein of *Staphylococcus aureus*, a bacterial antiinflammatory agent', *Journal of Experimental Medicine*, 199, 687–695

Deisenhofer, J. (1981) 'Crystallographic refinement and atomic models of a human Fc fragment and its complex with fragment B of protein A from *Staphylococcus aureus* at 2.9- and 2.8-A resolution', *Biochemistry*, 20, 2361–2370

DeLeo, F. R. and Chambers, H. F. (2009) 'Re-emergence of antibiotic-resistant *Staphylococcus aureus* in the genomics era', *The Journal of Clinical Investigation*, 119, 2464-2474

DeLeo, F. R., Otto, M., Kreiswirth, B. N. and Chambers, H. F. (2010) 'Community-associated methicillin-resistant *Staphylococcus aureus*', *Lancet*, 375, 1557–1568

DeMali, K. A., Wennerberg, K. and Burridge, K. (2003) 'Integrin signalling to the actin cytoskeleton', *Current Opinion in Cell Biology*, 15, 572–582

Denis, O., Deplano, A., De Beenhouwer, H., Hallin, M., Huysmans, G., Garrino, M. G., Glupczynski, Y., Malaviolle, X., Vergison, A. and Struelens, M. J. (2005) 'Polyclonal emergence and importation of community-acquired methicillin-resistant *Staphylococcus aureus* strains harbouring Panton-Valentine leucocidin genes in Belgium', *Journal of Antimicrobial Chemotherapy*, 56, 1103-1106

Denyer, S. P., Hodges, N. A. and Gorman, S. P. (2004) *Hugo and Russell's: Pharmaceutical microbiology*, 7th edn. Blackwell Science Ltd, Oxford UK

Department of Health Expert Advisory Committee on Antimicrobial Resistance and Healthcare Associated Infection (ARHAI) (2014) Implementation of modified admission MRSA screening guidance for NHS

Deshmukh, H. S., Hamburger, J. B., Ahn, S. H., McCafferty, D. G., Yang, S. R., Fowler, D. G. Jr. (2009) 'Critical role of NOD2 in regulating the immune response to *Staphylococcus aureus*', *Infection and Immunity*, 77, 1376-1382

Deurenberg, R. H. and Stobberingh, E. E. (2008) 'The evolution of *Staphylococcus aureus', Infection, Genetics, and Evolution*, 8, 747-763

Deurenberg, R. H. and Stobberingh, E. E. (2009) 'The molecular evolution of hospital- and community-associated methicillin-resistant *Staphylococcus aureus*', *Current Molecular Medicine*, 9, 100-115

Devriese, L. A. (1984) 'A simplified system for biotyping *Staphylococcus aureus* strains isolated from animal species', *Journal of Applied Bacteriology*, 56, 215–220

Devriese, L. A., Van Damme, L. R., and Fameree, L. (1972) 'Methicillin (cloxacillin)-resistant *Staphylococcus aureus* strains isolated from bovine mastitis cases', *Zentralbl. Veterinärmed.* 19, 598–605

Diekema, D. J., Pfaller, M. A, Schmitz, F. J., Smayevsky, J., Bell, J., Jones, R. N., Beach, M. and the SENTRY Participants Group (2001) 'Survey of infections due to Staphylococcus species: frequency of occurrence and antimicrobial susceptibility of isolates collected in the United States, Canada, Latin America, Europe, and the Western Pacific region for the SENTRY Antimicrobial Surveillance Program, 1997-1999', *Clinical Infectious Diseases*, 32(Suppl 2), S114-132

Diep, B. A., Carleton, H. A., Chang, R. F., Sensabaugh, G. F., Perdreau-Remington, F. (2006) 'Roles of 34 virulence genes in the evolution of hospital- and community-associated strains of methicillin-resistant *Staphylococcus aureus*', *Journal of Infectious Diseases*, 193, 1495–1503

Diep, B. A., Perdreau-Remington, F. and Sensabaugh, G. F. (2003) 'Clonal characterization of *Staphylococcus aureus* by multilocus restriction fragment typing, a rapid screening approach for molecular epidemiology', *Journal of Clinical Microbiology*, 41, 4559–4564

Dinges, M. M., Orwin, P. M. and Schlievert, P. M. (2000) 'Exotoxins of *Staphylococcus aureus*', *Clinical Microbiology Reviews*, 13, 16 –34

Dordel, J., Kim, C., Chung, M., Pardos de la Gándara, M., Holden, M. T., Parkhill, J., de Lencastre, H., Bentley, S. D. and Tomasz, A. (2014) 'Novel determinants of antibiotic resistance: identification of mutated loci in highly methicillin-resistant subpopulations of methicillin-resistant *Staphylococcus aureus*', *MBio*, 5, e01000

Dou, J. L., Jiang. Y. W., Xie, J. Q. and Zhang, X. G. (2015) 'New Is Old, and Old Is New: Recent Advances in Antibiotic-Based, Antibiotic-Free and Ethnomedical Treatments against Methicillin-Resistant *Staphylococcus aureus* Wound Infections', *International Journal of Molecular Sciences*, 17 (5)

Drevets, D. A., Canono, B. P., Leenen, P. J. and Campbell, P. A (1994) 'Gentamicin kills intracellular *Listeria Monocytogenes', Infection and Immunity*, 62, 2222–2228

Drew, R. H., Perfect, J. R., Srinath, L., Kurkimilis, E., Dowzicky, M. and Talbot, G. H. for the Synercid Emergency-Use Study Group (2000) 'Treatment of methicillin-resistant *Staphylococcus aureus* infections with quinupristin–dalfopristin in patients intolerant of or failing prior therapy', *Journal of Antimicrobial Chemotherapy*, 46, 775–784

Dryden, M. S. (2010) 'Complicated skin and soft tissue infection', *Journal of Antimicrobial Chemotherapy*, 65 Suppl 3: iii35–44

Duerden, B. I. (2012) 'MRSA: why have we got it and can we do anything about it?', *Eye*, 26, 218–221

Duerden, B., Fry, C., Johnson, A. P. and Wilcox, M. H. (2015) 'The control of methicillin-resistant *Staphylococcus aureus* blood stream infections in England, *Open Forum Infectious Diseases*, 2, ofv035

Duplessis, C. and Crum-Cianflone, N. F. (2011) 'Ceftaroline: a new cephalosporin with activity against methicillin-resistant *Staphylococcus aureus* (MRSA)', Clin Med Rev Ther, 3:a2466

Dziewanowska, K., Carson, A. R., Patti, J. M., Deobald, C. F., Bayles, K. W. and Bohach, G. A. (2000) 'Staphylococcal fibronectin binding protein interacts with heat shock protein 60 and integrins: role in internalization by epithelial cells', Infection and Immunity, 68, 6321–6328

Dziewanowska, K., Patti, J. M., Deobald, C. F., Bayles, K. W., Trumble, W. R. and Bohach, G. A. (1999) 'Fibronectin binding protein and host cell tyrosine kinase are required for internalization of *Staphylococcus aureus* by epithelial cells', *Infection and Immunity*, 67, 4673-4678

Economou, V. and Gousia, P. (2015) 'Agriculture and food animals as a source of antimicrobial-resistant bacteria', Infection and Drug Resistance, 8, 49–61

Edwards, A. M. and Massey, R. C. (2011) 'Invasion of human cells by a bacterial pathogen', *Journal of Visualized Experiments: JoVE*, (49), 2693

Egyir, B., Guardabassi, L., Monecke, S., Addo, K. K., Newman, M. J. and Larsen, A. R. (2015) 'Methicillin-resistant *Staphylococcus aureus* strains from Ghana include USA300', *Journal of Global Antimicrobial Resistance*, 3, 26–30

El Kholy, A., H. Baseem, G. S. Hall, G. W. Procop, and Longworth, D. L. (2003) 'Antimicrobial resistance in Cairo, Egypt 1999–2000: a survey of five hospitals', *Journal of Antimicrobial Chemotherapy*, 51, 625–630

Emele, F. E., Izomoh, M. I. and Alufohai, E. (1999) 'Microorganisms associated with wound infection in Ekpoma, Nigeria', *West African Journal of Medicine*, 18, 97–100

Emerson, D., Agulto, L., Liu, H. and Liu, L. (2008) 'Identifying and characterizing bacteria in an era of genomics and proteomics', *BioScience*, 58, 10, 925–936

Emori, T. G. and R. P. Gaynes. (1993) 'An overview of nosocomial infections, including the role of the microbiology laboratory', *Clinical Microbiology Reviews,* 6, 428–442

Enoch, D. A., Bygott, J. M., Daly, M. and Karas, J. A. (2007) 'Daptomycin', *Journal of Infection*, 55, 205–213

Enright, M. C., Robinson, D. A., Randle, G., Feil, E. J., Grundmann, H. and Spratt, B. G. (2002) 'The evolutionary history of methicillin-resistant *Staphylococcus aureus* (MRSA)', *Proceedings of the National Academy of Science U S A*, 99, 7687–7692

Esen, M., Schreiner, B., Jendrossek, V., Lang, F., Fassbender, K., Grassmé, H. and Gulbins E. (2001) 'Mechanisms of *Staphylococcus aureus* induced apoptosis of human endothelial cells', *Apoptosis*, 6, 431–439

Esimone C.O., Nworu C.S. and Udeogaranya O.P. (2007) 'Utilization of antimicrobial agents with and without prescription by out-patients in selected pharmacies in South-eastern Nigeria', *Pharmacy World and Science*, 29, 655-660

Essmann, F., Bantel, H., Totzke, G., Engels, I.H., Sinha, B., Schulze-Osthoff, K., and Jänicke, R.U. (2003) '*Staphylococcus aureus* α-toxin-induced cell death: predominant necrosis despite apoptotic caspase activation', *Cell Death and Differentiation*, 10, 1260–1272

Etebu, E. and Arikekpar, I. (2016) 'Antibiotics: classification and mechanisms of action with emphasis on molecular perspectives', *Int J Appl Microbiol Biotechnol Res,* 4, 90–101

Etz, H., Minh, D. B., Henics, T., Dryla, A., Winkler, B., Triska, C., Boyd, A. P., Sollner, J., Schmidt, W., von Ahsen, U., Buschle, M., Gill, S. R., Kolonay, J., Khalak, H., Fraser, C. M., von Gabain, A., Nagy, E. and Meinke, A. (2002) 'Identification of in vivo expressed vaccine candidate antigens from *Staphylococcus aureus*', *Proceedings of the National Academy of Sciences of the United States of America*, 99, 6573-6578

European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (2003) 'Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution', *Clinical Microbiology and Infection*, 9, ix–xx

European Committee for Antimicrobial Susceptibility Testing (EUCAST) (2017) European Committee on Antimicrobial Susceptibility Testing breakpoint tables for interpretation of MICs and zone diameters, version 7.1. http://www.eucast.org/fileadmin/src/media/PDFs/EUCAST_files/Breakpoint_tables/v_7.1_Breakpoint_Tables.pdf

Evans, A. S. (1998) 'Epidemiological concepts', In: Evans, A. S. and Brachman, P. S. (eds.) *Bacterial infections of humans*, New York, N.Y: Plenum Medical Book Company, pp. 3-64

Eybpoosh, S., Haghdoost, A. A., Mostafavi, E., Bahrampour, A., Azadmanesh, K. and Zolala, F. (2017) 'Molecular epidemiology of infectious diseases', *Electronic Physician*, 9, 5149–5158

Falagas, M. E., Karageorgopoulos, D. E., Leptidis, J. and Korbila, I. P. (2013) 'MRSA in Africa: filling the global map of antimicrobial resistance', *PLoS One*, 8(7)

Fasihi, Y., Fooladi, S., Mohammadi, M. A., Emaneini, M. and Kalantar-Neyestanaki, D. (2017) 'The spa typing of methicillin-resistant *Staphylococcus aureus* isolates by High Resolution Melting (HRM) analysis', *Journal of Medical Microbiology*, 66, 1335–1337

FDA (2010) 'FDA drug safety communication: increased risk of death with Tygacil (tigecycline) compared to other antibiotics used to treat similar infections', http://www.fda.gov/Drugs/DrugSafety/ucm224370.htm

Fernández-Barat, L., Ben-Aicha, S., Motos, A., Vila, J., Marco, F., Rigol, M., Muñoz, L., Bassi, G. L., Ferrer, M. and Torres, A. (2018) 'Assessment of *in vivo* versus *in vitro* biofilm formation of clinical methicillin-resistant *Staphylococcus aureus* isolates from endotracheal tubes', *Scientific Reports*, 8, 11906

Figueiredo, A. M. and Ferreira, F. A. (2014) 'The multifaceted resources and microevolution of the successful human and animal pathogen methicillin-resistant *Staphylococcus aureus*', *Memórias do Instituto Oswaldo Cruz*, 109, 265-278

Filice, G. A., Nyman, J. A., Lexau, C., Lees, C. H., Bockstedt, L. A., Como-Sabetti, K., Lesher, L. J. and Lynfield, R. (2010) 'Excess costs and utilization associated with methicillin resistance for patients with *Staphylococcus aureus* infection', *Infection Control and Hospital Epidemiology*, 31, 365–373

Fink, S. L. and Cookson, B. T. (2005) 'Apoptosis, pyroptosis, and necrosis: mechanistic description of dead and dying eukaryotic cells', *Infection and Immunity*, 73, 1907-1916

Fischbach, M. A., Lin, H., Liu, D. R. and Walsh, C. T. (2006) 'How pathogenic bacteria evade mammalian sabotage in the battle for iron', *Nature Chemical Biology*, 2, 132–138

Fischer, U., Jänicke, R. U., and Schulze-Osthoff, K. (2003) 'Many cuts to ruin: a comprehensive update of caspase substrates', *Cell Death and Differentiation*, 10, 76–100

Flannagan, R. S., Cosío, G. and Grinstein, S. (2009) 'Antimicrobial mechanisms of phagocytes and bacterial evasion strategies', *Nature Reviews Microbiology*, 7, 355-366

Flannagan, R. S., Heit, B. and Heinrichs, D. E. (2015) 'Antimicrobial mechanisms of macrophages and the immune evasion strategies of *Staphylococcus aureus*, *Pathogens (Basel, Switzerland)*, 4, 826–868

Flannagan, R. S., Heit, B., Heinrichs, D. E. (2016) 'Intracellular replication of *Staphylococcus aureus* in mature phagolysosomes in macrophages precedes host cell death, and bacterial escape and dissemination', Cellular Microbiology, 18, 514–535

Fleischer, B., and Schrezenmeier, H. (1988) 'T cell stimulation by staphylococcal enterotoxins: clonally variable response and requirement for major histocompatibility complex class II molecules on accessory or target cells', *Journal of Experimental Medicine,* 167, 1697–1707

Flemming, H. C., Wingender, J., Szewzyk, U., Steinberg, P., Rice, S. A. and Kjelleberg, S. (2016) 'Biofilms: an emergent form of bacterial life', *Nature Reviews Microbiology*, 14, 563-575

Flieger, A., Frischknecht, F., Häcker, G., Hornef, M. W. and Pradel, G. (2018) 'Pathways of host cell exit by intracellular pathogens', *Microbial Cell (Graz, Austria*), 5, 525–544

Florescu, I., Beuran, M., Dimov, R. Razbadauskas, A., Bochan, M., Fichev, G., Dukart, G., Babinchak, T., Cooper, C. A., Ellis-Grosse, E. J., Dartois, N. and Gandjini, H. on behalf of the 307 study group (2008) 'Efficacy and safety of tigecycline compared with vancomycin or linezolid for treatment of serious infections with methicillin-resistant *Staphylococcus aureus* or vancomycin-resistant enterococci: a phase 3, multicentre, double-blind, randomized study', *Journal of Antimicrobial Chemotherapy*, 62 Suppl 1: i17–28.

Flusberg, D. A. and Sorger, P. K. (2015) 'Surviving apoptosis: life-death signaling in single cells', *Trends in Cell Biology*, 25, 446–458

Fong, I. W. and Drlica, K. (2008) *Antimicrobial resistance and implications for the 21st century* (Eds.), Springer Science+Business Media, LLC: New York

Fonseca, A. B., Simon, M. D., Cazzaniga, R. A., de Moura, T. R., de Almeida, R. P., Duthie, M. S., Reed, S. G. and de Jesus, A. R. (2017) 'The influence of innate and adaptative immune responses on the differential clinical outcomes of leprosy', *Infectious Diseases of Poverty*, 6, 5

Foster, T. J. (2017) 'Antibiotic resistance in *Staphylococcus aureus:* Current status and future prospects', *FEMS Microbiology Reviews*, 41, 430–449

Foster, T. J. and Hook, M. (1998) 'Surface protein adhesins of *Staphylococcus aureus*', *Trends in Microbiology*, 6, 484–488

Foster, T. J., Geoghegan, J. A., Ganesh, V. K. and Hook, M. (2014) 'Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*', *Nature Reviews Microbiology*, 12, 49–62

Foster, T.J. (2004) 'Immune evasion by staphylococci', *The Journal of Clinical Investigation,* 114, 1693-1696

Foster, T.J. (2005) 'The *Staphylococcus aureus* superbug', *Nature Reviews Microbiology,* 3, 948–958

Fournier, G. P., Andam, C. P. and Gogarten, J. P. (2015) 'Ancient horizontal gene transfer and the last common ancestors', *BMC Evolutionary Biology,* 15, 70

Fournier, P., Dubourg, G. and Raoult, D. (2014) 'Clinical detection and characterization of bacterial pathogens in the genomics era', *Genome Medicine*, 6, 114

Fowler, T., Wann, E. R., Joh, D., Johansson, S., Foster, T. J. and Hook M. (2000) 'Cellular invasion by *Staphylococcus aureus* involves a fibronectin bridge between the bacterial fibronectin-binding MSCRAMMs and host cell beta1 integrins', *European Journal of Cell Biology*, 79, 672–679

Fowler, V. G. Jr, Boucher, H. W., Corey, G. R., Abrutyn, E., Karchmer, A. W., Rupp, M. E., Levine, D. P., Chambers, H. F., Tally, F. P., Vigliani, G. A., Cabell, C. H., Link, A. S., DeMeyer, I., Filler, S. G., Zervos, M., Cook, P., Parsonnet, J., Bernstein, J. M., Price, C. S., Forrest, G. N., Fätkenheuer, G., Gareca, M., Rehm, S. J., Brodt, H. R., Tice, A. and Cosgrove, S. E.; *S. aureus* Endocarditis and Bacteremia Study Group (2006) 'Daptomycin versus standard therapy for bacteremia and endocarditis caused by *Staphylococcus aureus'*, *New England Journal of Medicine*, 355, 653–665

Fowler, V. G. Jr., Miro, J. M., Hoen, B., Cabell, C. H., Abrutyn, E., Rubinstein, E., Corey, G. R., Spelman, D., Bradley, S. F., Barsic, B., Pappas, P. A., Anstrom, K. J., Wray, D., Fortes, C. Q., Anguera, I., Athan, E., Jones, P., van der Meer, J. T., Elliott, T. S., Levine, D. P. and Bayer, A. S.; ICE Investigators (2005) '*Staphylococcus aureus* endocarditis: a consequence of medical progress', *Journal of the American Medical Association*, 293, 3012–3021

Foxman, B. and Riley, L. (2001) 'Molecular epidemiology: focus on infection', *American Journal of Epidemiology*, 153, 1135-1141

Fraunholz, M. and Sinha, B. (2012) 'Intracellular *Staphylococcus aureus*: live-in and let die', *Frontiers in Cellular and Infection Microbiology*, 2, 43

Frei, E., Hodgkiss-Harlow, K., Rossi, P. J., Edmiston, C. E. Jr. and Bandyk, D. F. (2011) 'Microbial pathogenesis of bacterial biofilms: a causative factor of vascular surgical site infection', *Vascular and Endovascular Surgery*, 45, 688-696

French, G. L. (2006) 'Bactericidal agents in the treatment of MRSA infections—the potential role of daptomycin', *Journal of Antimicrobial Chemotherapy*, 58, 1107–1117

Fridkin, S. K., Hageman, J. C., Morrison, M., Sanza, L. T., Como-Sabetti, K., Jernigan, J. A., Harriman, K., Harrison, L. H., Lynfield, R. and Farley, M. M.; Active Bacterial Core Surveillance Program of the Emerging Infections Program Network (2005) 'Methicillin-resistant *Staphylococcus aureus Disease in Three Communities'*, *New England Journal of Medicine*, 352, 1436–1444

Friedrich, N., Hagedorn, M., Soldati-Favre, D., and Soldati, T. (2012) 'Prison break: pathogens' strategies to egress from host cells', *Microbiology and Molecular Biology Reviews: MMBR*, 76, 707–720

Fu, M. S., Coelho, C., De Leon-Rodriguez, C. M., Rossi, D., Camacho, E., Jung, E. H., Madhura, K. and Casadevall, A. (2018) '*Cryptococcus neoformans* urease affects the outcome of intracellular pathogenesis by modulating phagolysosomal pH', *PLoS Pathogens*, *14*, e1007144

Fuchs, P. C., Barry, A. L. and Brown, S. D. (2001) 'Interactions of quinupristin-dalfopristin with eight other antibiotics as measured by time-kill studies with 10 strains of *Staphylococcus aureus* for which quinupristin-dalfopristin alone was not bactericidal', *Antimicrobial Agents and Chemotherapy*, 45, 2662-2665

Fuchs, Y. and Steller, H. (2015) 'Live to die another way: modes of programmed cell death and the signals emanating from dying cells', *Nature Reviews Molecular Cell Biology*, 16, 329–344

Fuda, C., M. Suvorov, Q. Shi, D. Hesek, M. Lee, and S. Mobashery (2007) 'Shared functional attributes between the *mecA* gene product of *Staphylococcus sciuri* and penicillin-binding protein 2a of methicillin-resistant *Staphylococcus aureus', Biochemistry*, 46, 8050-8057

Galluzzi, L., Maiuri, M. C., Vitale, I., Zischka, H., Castedo, M., Zitvogel, L. and Kroemer, G. (2007) 'Cell death modalities: classification and pathophysiological implications', *Cell Death and Differentiation*, 14, 1237–1243

Galluzzi, L., Vitale, I., Aaronson, S., Abrams, J. M., Adam, D., Agostinis, P., Alnemri, E. S., Altucci, L., Amelio, I., Andrews, D. W., Annicchiarico-Petruzzelli, M., Antonov, A. V., Arama, E., Baehrecke, E. H., Barlev, N. A., Bazan, N. G., Bernassola, F., Bertrand, M. J. M., Bianchi, K., Blagosklonny, M. V., Blomgren, K., Borner, C., Boya, P., Brenner, C., Campanella, M., Candi, E., Carmona-Gutierrez, D., Cecconi, F., Chan, F. K., Chandel, N. S., Cheng, E. H., Chipuk, J. E., Cidlowski, J. A., Ciechanover, A., Cohen, G. M., Conrad, M., Cubillos-Ruiz, J. R., Czabotar, P. E., D'Angiolella, V., Dawson, T. M., Dawson, V. L., De Laurenzi, V., De Maria, R., Debatin, K. M., DeBerardinis, R. J., Deshmukh, M., Di Daniele, N., Di Virgilio, F., Dixit, V. M., Dixon, S. J., Duckett, C. S., Dynlacht, B. D., El-Deiry, W. S., Elrod, J. W., Fimia, G. M., Fulda, S., García-Sáez, A. J., Garg, A. D., Garrido, C., Gavathiotis, E., Golstein, P., Gottlieb, E., Green, D. R., Greene, L. A., Gronemeyer, H., Gross, A., Hajnoczky, G., Hardwick, J. M., Harris, I. S., Hengartner, M. O., Hetz, C., Ichijo, H., Jäättelä, M., Joseph, B., Jost, P. J., Juin, P. P., Kaiser, W. J., Karin, M., Kaufmann, T., Kepp, O., Kimchi, A., Kitsis, R. N., Klionsky, D. J., Knight, R. A., Kumar, S., Lee, S. W., Lemasters, J. J., Levine, B., Linkermann, A., Lipton, S. A., Lockshin, R. A., López-Otín, C., Lowe, S. W., Luedde, T., Lugli, E., MacFarlane, M., Madeo, F., Malewicz, M,, Malorni, W., Manic, G., Marine, J. C., Martin, S. J., Martinou, J. C., Medema, J. P., Mehlen, P., Meier, P., Melino, S., Miao, E. A., Molkentin, J. D., Moll, U. M., Muñoz-Pinedo, C., Nagata, S., Nuñez, G., Oberst, A., Oren, M., Overholtzer. M., Pagano. M., Panaretakis, T., Pasparakis, M., Penninger, J. M., Pereira, D. M., Pervaiz, S., Peter, M. E., Piacentini, M., Pinton, P., Prehn, J. H. M., Puthalakath, H., Rabinovich, G. A., Rehm, M., Rizzuto, R., Rodrigues, C. M. P., Rubinsztein, D. C., Rudel, T., Ryan, K., Sayan, E., Scorrano, L., Shao, F., Shi, Y., Silke, J., Simon, H. U., Sistigu, A., Stockwell, B. R., Strasser, A., Szabadkai, G., Tait, S. W. G., Tang, D., Tavernarakis, N., Thorburn, A., Tsujimoto, Y., Turk, B., Vanden Berghe, T.,

Vandenabeele, P., Vander Heiden, M. G., Villunger. A., Virgin. H. W., Vousden, K. H., Vucic, D., Wagner, E. F., Walczak, H., Wallach, D., Wang, Y., Wells, J. A., Wood, W., Yuan, J., Zakeri, Z., Zhivotovsky, B., Zitvogel, L., Melino, G. and Kroemer, G. (2018) 'Molecular mechanisms of cell death: recommendations of the Nomenclature Committee on Cell Death 2018', *Cell Death and Differentiation*, 25, 486–541

Galluzzi, L., Vitale, I., Abrams, J. M., Alnemri, E. S., Baehrecke, E. H., Blagosklonny, M. V., Dawson, T. M., Dawson, V. L., El-Deiry, W. S., Fulda, S., Gottlieb, E., Green, D. R., Hengartner, M. O., Kepp, O., Knight, R. A., Kumar, S., Lipton, S. A., Lu, X., Madeo, F., Malorni, W., Mehlen, P., Nuñez, G., Peter, M. E., Piacentini, M., Rubinsztein, D. C., Shi, Y., Simon, H. U., Vandenabeele, P., White, E., Yuan, J., Zhivotovsky, B., Melino, G. and Kroemer, G. (2012) 'Molecular definitions of cell death subroutines: recommendations of the nomenclature committee on cell death 2012', *Cell Death and Differentiation*, 19, 107–120

Garaizar, J., Rementeria, A. and Porwollik, S. (2006) 'DNA microarray technology: a new tool for the epidemiological typing of bacterial pathogens', *FEMS Immunology and Medical Microbiology*, 47, 178-189

Garcia, L. G., Lemaire, S., Kahl, B. C., Becker, K., Proctor, R. A., Denis, O., Tulkens, P. M.and Van Bambeke, F. (2013) 'Antibiotic activity against small-colony variants of Staphylococcus aureus: review of in vitro, animal and clinical data', *Journal of Antimicrobial Chemotherapy*, 68, 1455–1464

Garcia-Alvarez, L., Holden, M. T., Lindsay, H., Webb, C. R., Brown, D. F., Curran, M. D., Walpole, E., Brooks, K., Pickard, D. J., Teale, C., Parkhill, J., Bentley, S. D., Edwards, G. F., Girvan, E. K., Kearns, A. M., Pichon, B., Hill, R. L., Larsen, A. R., Skov, R. L., Peacock, S. J., Maskell, D. J. and Holmes, M. A. (2011) 'Meticillin-resistant *Staphylococcus aureus* with a novel *mecA* homologue in human and bovine populations in the UK and Denmark: a descriptive study', *Lancet Infectious Diseases*, 11 595–603

Geisinger, E, Muir, T. W., Novick, R. P. and Bassler, B. L. (2009) 'agr receptor mutants reveal distinct modes of inhibition by staphylococcal autoinducing peptides', *Proceedings of the National Academy of Sciences of the United States of America*, 106, 1216-1221

Gemmell, C. G., Edwards, D. I., Fraise, A. P., Gould, F. K., Ridgway, G. L. and Warren, R. E. on behalf of the Joint Working Party of the British Society for Antimicrobial Chemotherapy, Hospital Infection Society and Infection Control Nurses Association (2006) 'Guidelines for the prophylaxis and treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in the UK', *Journal of Antimicrobial Chemotherapy*, 57, 589–608

Genestier, A. L., Michallet, M. C., Prévost, G., Bellot, G., Chalabreysse, L., Peyrol, S., Thivolet, F., Etienne, J., Lina, G., Vallette, F. M., Vandenesch, F. and Genestier, L. (2005) '*Staphylococcus aureus* Panton-Valentine leukocidin directly targets mitochondria and induces Bax-independent apoptosis of human neutrophils', *Journal of Clinical Investigation*, 115, 3117–3127

Ghaznavi-Rad, E., Shamsudin M. N., Sekawi, Z., Khoon, L. Y., Aziz, M. N., Hamat, R. A. and Neela, V. (2010) 'Predominance and emergence of clones of hospital-acquired methicillin-resistant *Staphylococcus aureus* in Malaysia', *Journal of Clinical Microbiology*, 48, 867-872

Ghebremedhin, B., Olugbosi, M. O., Raji, M., Layer, F., Bakare, R. A., Konig, B. and Konig, W. (2009) 'Emergence of a community-associated methicillin-resistant *Staphylococcus aureus* with unique resistance profile in Southwest of Nigeria', *Journal of Clinical Microbiology*, 47, 2975-2980

Ghosh, R. and Pattison, J. S. (2018) 'Macroautophagy and chaperone-mediated autophagy in heart failure: the known and the unknown', *Oxidative Medicine and Cellular Longevity*, 8602041

Gibert, B. and Mehlen, P. (2015) 'Dependence receptors and cancer: addiction to trophic ligands', *Cancer Research*, 75, 5171–5175

Giese, B., Dittmann, S., Paprotka, K., Levin, K., Weltrowski, A., Biehler, D., Lam, T. T., Sinha, B. and Fraunholz, M. J. (2009) 'Staphylococcal alpha-toxin is not sufficient to mediate escape from phagolysosomes in upper-airway epithelial cells', *Infection and Immunity*, 77, 3611–3625

Giese, B., Glowinski, F., Paprotka, K., Dittmann, S., Steiner, T., Sinha, B. and Fraunholz, M. J. (2011) 'Expression of δ-toxin by *Staphylococcus aureus* mediates escape from phago-endosomes of human epithelial and endothelial cells in the presence of β-toxin', *Cellular Microbiology*, 13, 316-329

Gilbert, D. F., Erdmann, G., Zhang, X., Fritzsche, A., Demir, K., Jaedicke, A., Muehlenberg K, Wanker, E. E. and Boutros, M. (2011) 'A novel multiplex cell viability assay for high-throughput RNAi screening', *PloS One*, 6, e28338

Gillet, Y., Issartel, B., Vanhems, P., Fournet, J. C., Lina, G., Bes, M., Vandenesch, F., Piémont, Y., Brousse, N., Floret, D. and Etienne, J. (2002) 'Association between *Staphylococcus aureus* strains carrying gene for Panton-Valentine leukocidin and highly lethal necrotising pneumonia in young immunocompetent patients', *Lancet*, 359, 753-759

Gläser, R., Becker, K., von Eiff, C., Meyer-Hoffert, U. and Harder, J. (2014) 'Decreased susceptibility of *Staphylococcus aureus* small-colony variants toward human antimicrobial peptides', *Journal of Investigative Dermatology*, 134, 2347–2350

Goering, R. V., Shawar, R. M., Scangarella, N. E., O'Hara, F. P., Amrine-Madsen, H., West, J. M., Dalessandro, M., Becker, J. A., Walsh, S. L., Miller, L. A., van Horn, S. F., Thomas, E. S. and Twynholm, M. E. (2008) 'Molecular epidemiology of methicillin-resistant and methicillin-susceptible *Staphylococcus aureus* isolates from global clinical trials', *Journal of Clinical Microbiology*, 46, 2842-2847

Gomes-Fernandes, M., Laabei, M., Pagan, N., Hidalgo, J., Molinos, S., Villar Hernandez, R., Domínguez-Villanueva, D., Jenkins, A. T. A., Lacoma, A. and Prat, C. (2017) 'Accessory

gene regulator (Agr) functionality in *Staphylococcus aureus* derived from lower respiratory tract infections', *PloS One*, 12, e0175552

Gomez-Sanz, E., Schwendener, S., Thomann, A., Gobeli Brawand S. and Perreten V. (2015) ,First staphylococcal cassette chromosome *mec* containing a *mecB*-carrying gene complex independent of transposon Tn6045 in a *Macrococcus caseolyticus* isolate from a canine infection', *Antimicrobial Agents and Chemotherapy*, 59, 4577–4583

Gonzalez, C., Rubio, M., Romero-Vivas, J., Gonzalez, M., and Picazo, J. J. (1999) 'Bacteremic pneumonia due to *Staphylococcus aureus*: a comparison of disease caused by methicillin-resistant and methicillin-susceptible organisms', *Clinical Infectious Diseases*, 29, 1171–1177

Gonzalez, C., Rubio, M., Romero-Vivas, J., Gonzalez, M., and Picazo, J. J. (2003) '*Staphylococcus aureus* bacteremic pneumonia: differences between community and nosocomial acquisition', *International Journal of Infectious Diseases*, 7, 102–108

Goodyear,C. S. and Silverman, G. J. (2003) 'Death by a B cell superantigen: *in vivo* VH-targeted apoptotic supraclonal B cell deletion by a staphylococcal toxin', *Journal of Experimental Medicine*, 197, 1125–1139

Gordon, C. P., Williams, P. and Chan, W. C. (2013) 'Attenuating *Staphylococcus aureus* virulence gene regulation: a medicinal chemistry perspective', *Journal of Medicinal Chemistry*, 56, 1389–1404

Gordon, J. R. and Lowy F.D. (2008) 'Pathogenesis of methicillin-resistant *Staphylococcus aureus* infection', *Clinical Infectious Diseases*, 46, S350–S359

Gordon, N. C., Pichon, B., Golubchik, T., Wilson, D. J., Paul, J., Blanc, D. S., Cole, K., Collins, J., Cortes, N., Cubbon, M., Gould, F. K., Jenks, P. J., Llewelyn, M., Nash, J. Q., Orendi, J. M., Paranthaman, K., Price, J. R., Senn, L., Thomas, H., Wyllie, S., Crook, D. W., Peto, T. E. A., Walker, A. S. and Kearns, A. M. (2017) 'Whole-Genome Sequencing Reveals the Contribution of Long-Term Carriers in *Staphylococcus aureus* Outbreak Investigation', *Journal of Clinical Microbiology*, 55, 2188-2197

Gould, F. K., Brindle, R., Chadwick, P. R., Fraise, A. P., Hill, S., Nathwani, D., Ridgway, G. L., Spry, M. J. and Warren, R. E. MRSA Working Party of the British Society for Antimicrobial Chemotherapy (2009) 'Guidelines (2008) for the prophylaxis and treatment of methicillin-resistant Staphylococcus aureus (MRSA) infections in the United Kingdom', *Journal of Antimicrobial Chemotherapy*, 63, 849–861

Gould, I. M., David, M. Z., Esposito, S., Garau, J., Lina, G., Mazzei, T., Peters, G. (2012) 'New insights into methicillin resistant *Staphylococcus aureus* pathogenesis, treatment and resistance', *International Journal of Antimicrobial Agents*, 39, 96–104

Gould, I. M., Reilly, J., Bunyan, D. and Walker, A. (2010) 'Costs of healthcare-associated methicillin-resistant *Staphylococcus aureus* and its control', *Clinical Microbiology and Infection*, 16, 1721–1728

Gray, B., Hall, P. and Gresham, H. (2013) 'Targeting agr- and agr-like quorum sensing systems for development of common therapeutics to treat multiple Gram-positive bacterial infections', *Sensors*, 13, 5130-5166

Green, B. N., Johnson, C. D., Egan, J. T., Rosenthal, M., Griffith, E. A. and Evans, M. W. (2012) 'Methicillin-resistant *Staphylococcus aureus*: an overview for manual therapists', *Journal of Chiropractic Medicine*, 11, 64-76

Green, D. R. and Llambi, F. (2015) 'Cell Death Signaling', *Cold Spring Harbor Perspectives in Biology*, 7:a006080.

Green, D. R., Ferguson, T., Zitvogel, L. and Kroemer, G. (2009) 'Immunogenic and tolerogenic cell death', *Nature Reviews Immunology*, 9, 353-363

Green, D. R., Oguin, T. H. and Martinez, J. (2016) 'The clearance of dying cells: table for two', *Cell Death and Differentiation*, 23, 915-926.

Greer, R. B. I. and Rosenberg, A. E. (1993) 'Case records of the Massachusetts general hospital case 6', *New England Journal of Medicine*, 328, 422-428

Grosz, M., Kolter, J., Paprotka, K., Winkler, A. C., Schäfer, D., Chatterjee, S. S., Geiger, T., Wolz, C., Ohlsen, K., Otto, M., Rudel, T., Sinha, B. and Fraunholz, M. (2013) Cytoplasmic replication of *Staphylococcus aureus* upon phagosomal escape triggered by phenol-soluble modulin α', *Cellular Microbiology*, 16, 451–465

Grundmann, H. (2014) 'Towards a global antibiotic resistance surveillance system: a primer for a roadmap', *Upsala Journal of Medical Sciences*, 119, 87–95

Grundmann, H., Aanensen, D. M., van den Wijngaard, C. C., Spratt, B. G., Harmsen, D., and Friedrich, A. W. (2010) 'Geographic distribution of *Staphylococcus aureus* causing invasive infections in Europe: a molecular-epidemiological analysis', *PLoS Medicine*, 7(1)

Grundmeier, M., Hussain, M., Becker, P., Heilmann, C., Peters, G. and Sinha B. (2004) 'Truncation of fibronectin-binding proteins in *Staphylococcus aureus* strain Newman leads to deficient adherence and host cell invasion due to loss of the cell wall anchor function', Infection and Immunity, 72, 7155–7163

Gu, B., Kelesidis, T., Tsiodras, S., Hindler, J. and Humphries, R. M. (2013) 'The emerging problem of linezolid-resistant *Staphylococcus*', *Journal of Antimicrobial Chemotherapy*, 68, 4–11.

Gu, F. F., Han, L. Z., Chen, X., Wang, Y. C., Shen, H., Wang, J. Q., Tang, J., Zhang, J. and Ni, Y. X. (2015) 'Molecular characterization of *Staphylococcus aureus* from surgical site

infections in orthopedic patients in an orthopedic trauma clinical medical center in Shanghai', *Surgical infections (Larchmt),* 16, 97–104

Guerra, F. E., Borgogna, T. R., Patel, D. M., Sward, E. W. and Voyich, J. M. (2017) 'Epic immune battles of history: neutrophils vs. *Staphylococcus aureus*', *Frontiers in Cellular and Infection Microbiology*, *7*, 286

Hallin, M., Deplano, A., Denis, O., De Mendonca, R., De Ryck, R. and Struelens, M. J. (2007) 'Validation of pulsed-field gel electrophoresis and spa typing for long-term, nationwide epidemiological surveillance studies of *Staphylococcus aureus* infections', *Journal of Clinical Microbiology,* 45, 127-133

Hamdan-Partida, A., Sainz-Espuñes, T., and Bustos-Martínez, J. (2010) 'Characterization and persistence of *Staphylococcus aureus* strains isolated from the anterior nares and throats of healthy carriers in a Mexican community', *Journal of Clinical Microbiology*, 48, 1701–1705

Hanahan, D. and Weinberg, R. A. (2011) 'Hallmarks of cancer: the next generation', *Cell*, 144, 646-674

Hanaki, H., Kuwahara-Arai, K., Boyle-Vavra, S., Daum, R. S., Labischinski, H. and Hiramatsu, K. (1998) 'Activated cell-wall synthesis is associated with vancomycin resistance in methicillin-resistant *Staphylococcus aureus* clinical strains Mu3 and Mu50', *Journal of Antimicrobial Chemotherapy*, 42, 199–209.

Hanaki, H., Labischinski, H., Inaba, Y., Kondo, N., Murakami, H. and Hiramatsu, K. (1998) 'Increase in glutamine-non-amidated muropeptides in the peptidoglycan of vancomycin-resistant *Staphylococcus aureus* strain Mu50', *Journal of Antimicrobial Chemotherapy,* 42, 315–320 (b)

Hanberger, H., Walther, S., Leone, M., Barie, P. S., Rello, J., Lipman, J., Marshall, J. C., Anzueto, A., Sakr, Y., Pickkers, P., Felleiter, P., Engoren, M. and Vincent, J. L.; EPIC II Group of Investigators. (2011) 'Increased mortality associated with methicillin-resistant *Staphylococcus aureus* (MRSA) infection in the intensive care unit: results from the EPIC II Study', *International Journal of Antimicrobial Agents,* 38, 331–335

Hanslick, J. L., Lau, K., Noguchi, K. K., Olney, J. W., Zorumski, C. F., Mennerick, S., and Farber, N. B. (2009) 'Dimethyl sulfoxide (DMSO) produces widespread apoptosis in the developing central nervous system', *Neurobiology of. Disease*, 34, 1–10

Hanssen, A. M. and Sollid, J. U. (2007) 'Multiple staphylococcal cassette chromosomes and allelic variants of cassette chromosome recombinases in *Staphylococcus aureus* and coagulase-negative staphylococci from Norway', *Antimicrobial Agents and Chemotherapy*, 51, 1671–1677

Hanssen, A. M., and Sollid E. J. U. (2006) 'SCCmec in Staphylococci: Genes on the move', FEMS Immunology and Medical Microbiology, 46, 8–20

Harraghy, N., Hussain, M., Haggar, A., Chavakis, T., Sinha, B., Herrmann, M. and Flock, J. I. (2003) 'The adhesive and immunomodulating properties of the multifunctional *Staphylococcus aureus* protein Eap', *Microbiology*, 149, 2701–2707

Harris, S. R., Cartwright, E. J., Török, M. E., Holden, M. T., Brown, N. M., Ogilvy-Stuart, A. L., Ellington, M. J., Quail, M. A., Bentley, S. D., Parkhill, J. and Peacock, S. J. (2013) 'Whole-genome sequencing for analysis of an outbreak of meticillin-resistant *Staphylococcus aureus*: a descriptive study', *The Lancet Infectious Diseases*, 13, 130–136

Harris, S. R., Feil, J., Holden, M. T., Quail, M. A., Nickerson, E. K., Chantratita, N., Gardete, S., Tavares, A., Day, N., Lindsay, J. A., Edgeworth, J. D., de Lencastre, H., Parkhill, J., Peacock, S. J. and Bentley, S. D. (2010) 'Evolution of MRSA during hospital transmission and intercontinental spread', *Science*, 327, 469-474

Harrison, E. M., Paterson, G. K., Holden, M. T., Ba, X., Rolo, J., Morgan, F. J., Pichon, B., Kearns, A., Zadoks, R. N., Peacock, S. J., Parkhill, J. and Holmes, M. A. (2014) 'A novel hybrid SCC*mec*-*mecC* region in *Staphylococcus sciuri*', *Journal of Antimicrobial Chemotherapy*, 69, 911–918

Harrison, E. M., Paterson, G. K., Holden, M. T., Morgan, F. J., Larsen, A. R., Petersen, A., Leroy, S., De Vliegher, S., Perreten, V., Fox, L. K., Lam, T. J., Sampimon, O. C., Zadoks, R. N., Peacock, S. J., Parkhill, J. and Holmes, M. A. (2013) 'A *Staphylococcus xylosus* isolate with a new *mecC* allotype', *Antimicrobial Agents and Chemotherapy*, 57, 1524–1528

Haslinger, B., Strangfeld, K., Peters, G., Schulze-Osthoff, K. and Sinha, B. (2003) '*Staphylococcus aureus* alpha-toxin induces apoptosis in peripheral blood mononuclear cells: role of endogenous tumour necrosis factor-alpha and the mitochondrial death pathway', *Cellular Microbiology*, 5, 729–741

Haslinger-Löffler, B., Kahl, B. C., Grundmeier, M., Strangfeld, K., Wagner, B., Fischer, U., Cheung, A. L., Peters, G., Schulze-Osthoff, K. and Sinha B. (2005) 'Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis in endothelial cells', *Cellular Microbiology*, 7, 1087–1097

Hatcher, H. C., Singh, R. N., Torti, F. M. and Torti, S. V. (2009) 'Synthetic and natural iron chelators: therapeutic potential and clinical use', *Future Medicinal Chemistry*, 1, 1643-1670

Hauck, C. R. and Ohlsen, K. (2006) 'Sticky connections: extracellular matrix protein recognition and integrin-mediated cellular invasion by *Staphylococcus aureus*', *Current Opinion in Microbiology*, 9, 5–11

He, W., Chen, H., Zhao, C., Zhang, F., Li, H., Wang, Q., Wang, X. and Wang, H. (2013) 'Population structure and characterisation of *Staphylococcus aureus* from bacteraemia at multiple hospitals in China: association between antimicrobial resistance, toxin genes and genotypes', *International Journal of Antimicrobial Agents*, 42, 211-219

Heli, H., Mirtorabi, S. and Karimian, K. (2011) 'Advances in iron chelation: an update', *Expert Opinion on Therapeutic Patents*, 21, 819–856

Henderson, A. and Nimmo, G. R. (2018) 'Control of healthcare- and community-associated MRSA: recent progress and persisting challenges', *British Medical Bulletin*, 125:25

Henrici, A. T. (1934) 'Infection', In: *The biology of bacteria*, D.C. Boston, Mass: Heath and Co., pp. 230–241

Hider, R. C. and Kong, X. (2010) 'Chemistry and biology of siderophores', *Natural Product Reports*, 27, 637–657

Hider, R. C. and Zhou, T. (2005) 'The design of orally active iron chelators', *Annals of the New York Academy of Sciences*, 1054, 141–154

Hill, C. (2012) 'Virulence or niche factors: what's in a name?', *Journal of Bacteriology*, 194, 5725–5727

Hiramatsu, K. (1995) 'Molecular evolution of MRSA', *Microbiology and Immunology*, 39, 531-543

Hiramatsu, K., Cui, L., Kuroda, M. and Ito, T. (2001) 'The emergence and evolution of methicillin-resistant *Staphylococcus aureus'*, *Trends in Microbiology*, 9, 486–493

Hiramatsu, K., Hanaki, H., Ino, T., Yabuta, K., Oguri, T. and Tenover, F. C. (1997) 'Methicillin-resistant *Staphylococcus aureus* clinical strain with reduced vancomycin susceptibility', *Journal of Antimicrobial Chemotherapy*, 40, 135–136

Hiramatsu, K., Ito, T., Tsubakishita, S., Sasaki, T., Takeuchi, F., Morimoto, Y., Katayama, Y., Matsuo, M., Kuwahara-Arai, K., Hishinuma, T. and Baba, T. (2013) 'Genomic basis for methicillin resistance in *Staphylococcus aureus*', *Infection and Chemotherapy*, 45, 117-136

Hiramatsu, K., Katayama, Y., Matsuo, M., Sasaki, T., Morimoto, Y., Sekiguchi, A. and Baba, T. (2014) 'Multi-drug-resistant *Staphylococcus aureus* and future chemotherapy', *Journal of Infection and Chemotherapy*, 20, 593-601

Hiramatsu, K., Katayama, Y., Yuzawa, H. and Ito, T. (2002) 'Molecular genetics of methicillin-resistant *Staphylococcus aureus'*, *International Journal of Microbiology*, 292, 67-74

Hoang, S., Georget, A., Asselineau, J., Venier, A-G., Leroyer, C., Rogues, A. M. and Thiebaut, R. (2018) 'Risk factors for colonization and infection by *Pseudomonas aeruginosa* in patients hospitalized in intensive care units in France', *PLoS ONE*, 13: e0193300

Hoffmann, C., Berking, A., Agerer, F., Buntru, A., Neske, F., Chhatwal, G. S., Ohlsen, K. and Hauck, C. R. (2010) 'Caveolin limits membrane microdomain mobility and integrin-mediated uptake of fibronectin-binding pathogens', *Journal of Cell Science*, 123, 4280–4291

Hoffmann, C., Ohlsen, K. and Hauck, C. R. (2011) 'Integrin-mediated uptake of fibronectin-binding bacteria," *European Journal of Cell Biology*, 90, 891–896

Holmes, N. E., Turnidge, J. D., Munckhof, W. J., Robinson, J. O., Korman, T. M., O'Sullivan, M. V., Anderson, T. L., Roberts, S. A., Gao, W., Christiansen, K. J., Coombs, G. W., Johnson, P. D. and Howden, B. P. (2011) 'Antibiotic choice may not explain poorer outcomes in patients with *Staphylococcus aureus* bacteremia and high vancomycin minimum inhibitory concentrations', *Journal of Infectious Disease*, 204, 340–347

Hooper, D. C. and Jacoby, G. A. (2015) 'Mechanisms of drug resistance: quinolone resistance', *Annals of the New York Academy of Sciences*, 1354, 12–31

Howden, B. P. (2014) 'Antibiotics and *Staphylococcus aureus*-more than meets the MIC', *Journal of molecular medicine (Berlin, Germany*, 92, 103–106

Hsu, D. I., Hidayat, L. K., Quist, R., Hindler, J., Karlsson, A., Yusof, A. and Wong-Beringer, A. (2008) 'Comparison of method-specific vancomycin minimum inhibitory concentration values and their predictability for treatment outcome of meticillin-resistant Staphylococcus aureus (MRSA) infections', *International Journal of Antimicrobial Agents*, 32, 378-385

http://spaserver.ridom.de

Hu, L. and Kopecko, D. J. (1999) '*Campylobacter jejuni* 81-176 associates with microtubules and dynein during invasion of human intestinal cells', *Infection and Immunity*, 67, 4171–4182

Hu, Q., Cheng, H., Yuan, W., Zeng, F., Shang, W., Tang, D., Xue, W., Fu, J., Zhou, R., Zhu, J., Yang, J., Hu, Z., Yuan, J., Zhang, X., Rao, Q., Li, S., Chen, Z., Hu, X., Wu, X. and Rao, X. (2015) 'Panton-Valentine Leukocidin (PVL)-positive health care-associated methicillin-resistant Staphylococcus aureus isolates are associated with skin and soft tissue infections and colonized mainly by infective PVL-encoding bacteriophages', *Journal of Clinical Microbiology*, 53, 67-72

Hübner, C., Hübner, N., Wegner, C. and Flessa, S. (2015) 'Impact of different diagnostic technologies for MRSA admission screening in hospitals – a decision tree analysis', *Antimicrobial Resistance and Infection Control*, 4, 50

Hultgren, O., Kopf, M. and Tarkowski, A. (1998) '*Staphylococcus aureus*-induced septic arthritis and septic death is decreased in IL-4-deficient mice: role of IL-4 as promoter for bacterial growth', *Journal of Immunology*, 160, 5082-5087

Hussain, M., Haggar, A., Peters, G., Chhatwal, G. S., Herrmann, M., Flock, J. I. and Sinha, B. (2008) 'More than one tandem repeat domain of the extracellular adherence protein of Staphylococcus aureus is required for aggregation, adherence, and host cell invasion but not for leukocyte activation', Infection and Immunity 76, 5615–5623

Hybiske, K. and Stephens, R. S. (2008) 'Exit strategies of intracellular pathogens', *Nature Reviews Microbiology*, 6, 99-110

Hynes, R. O. (1990) *Fibronectins*, New York Berlin Heidelberg: Springer

Ibrahim, A. S., Gebermariam, T., Fu, Y., Lin, L., Husseiny, M. I., French, S. W., Schwartz, J., Skory, C. D., Edwards, J. E. Jr. and Spellberg, B. J. (2007) 'The iron chelator deferasirox protects mice from mucormycosis through iron starvation', J*ournal of Clinical Investigation*, 117, 2649-2657

Igbeneghu, O. A. (2013) 'Knowledge and practices in the use of antibiotics among a group of Nigerian university students', *International Journal of Infection Control*, 9, i1

Ikeda, T. and Tazuke, S. (1983) 'Biologically active polycations: Antimicrobial activities of Poly[trialkyl(vinylbenzyl)ammonium chloride]-type polycations', *Macromolecular Rapid Communications*, 4, 459-461

Ikeda, T., Hirayama, H., Yamaguchi, H., Tazuke, S. and Watanabe, M. (1986) 'Polycationic biocides with pendant active groups-molecular weight dependence of antibacterial activity', *Antimicrobial Agents and Chemotherapy*, 30, 132-136

International Working Group on the Classification of Staphylococcal Cassette Chromosome Elements (IWG-SCC) (2009) 'Classification of staphylococcal cassette chromosome *mec* (SCC*mec*): guidelines for reporting novel SCC*mec* elements', *American Society for Microbiology*, 53, 4961–4967

Ito, T., Katayama, Y. and Hiramatsu, K. (1999) 'Cloning and nucleotide sequence determination of the entire *mec* DNA of pre-methicillin-resistant *Staphylococcus aureus* N315', *Antimicrobial Agents and Chemotherapy*, 43, 1449–1458

Ito, T., Katayama, Y., Asada, K., Mori, N., Tsutsumimoto, K., Tiensasitorn, C. and Hiramatsu, K. (2001) 'Structural comparison of three types of staphylococcal cassette chromosome *mec* integrated in the chromosome in methicillin-resistant *Staphylococcus aureus', Antimicrobial Agents and Chemotherapy*, 45, 1323–1336

Ito, T., Ma, X. X., Takeuchi, F., Okuma, K., Yuzawa, H. and Hiramatsu, K. (2004) 'Novel type V staphylococcal cassette chromosome *mec* driven by a novel cassette chromosome recombinase, *ccrC', Antimicrobial Agents and Chemotherapy*, 48, 2637–2651

Ito, T., Okuma, K., Ma, X.X., Yuzawa, H. and Hiramatsu, K. (2003) 'Insights on antibiotic resistance of *Staphylococcus aureus* from its whole genome: genomic island SCC', *Drug Resistance Updates*, 6, 41–52

Jabra-Rizk, M. A., Kong, E. F., Tsui, C., Nguyen, M. H., Clancy, C. J., Fidel, P. L. and Noverr, M. (2016) '*Candida albicans* pathogenesis: fitting within the host-microbe damage response framework', *Infection and Immunity*, 84, 2724–2739

Jakubovics, N. S., Shields. R. C., Rajarajan, N. and Burgess, J. G. (2013) 'Life after death: the critical role of extracellular DNA in microbial biofilms', *Letters in Applied Microbiology*, 57, 467—475

Jan-Roblero, J., Garcia-Gomez, E., Rodriquez-Martinez, S., Cancino-Diaz, M. E. and Cancino-Diaz, J. C. (2017) 'Surface Proteins of *Staphylococcus aureus',* Chapter of book *The Rise of Virulence and Antibiotic Resistance in Staphylococcus aureus*, Enany, S. and Alexander, L. E. C., Intech

Jarry, T. M., Memmi, G. and Cheung, A. L. (2008) 'The expression of alpha-haemolysin is required for *Staphylococcus aureus* phagosomal escape after internalisation in CFT-1 cells', *Cellular Microbiology*, 10, 1801-1814

Jenul, C. and Horswill, A. R. (2018) 'Regulation of *Staphylococcus aureus* virulence', *Microbiology Spectrum*, 6, 1

Jevons, M. P. and Parker, M. T. (1964) 'The evolution of new hospital strains of *Staphylococcus aureus*', *Journal of Clinical Pathology*, 17, 243–250

Jevons, M. P., Coe, A. W. and Parker, M. T. (1963) 'Methicillin resistance in staphylococci', *Lancet*, 1, 904–907

Ji, G., Beavis, R. and Novick, R. P. (1997) 'Bacterial interference caused by autoinducing peptide variants', *Science*, 276, 2027–2030

Ji, G., Beavis, R. C. and Novick, R. P. (1995) 'Cell density control of staphylococcal virulence mediated by an octapeptide pheromone', *Proceedings of the National Academy of Sciences of the United States of America*, 92, 12055–12059

Jiang, X. and Pace, J. L. (2006) 'Microbial biofilms', pp.3–19, in *Biofilms, Infection and Antimicrobial Therapy*, Pace, J. L., Rupp, M. E. and Finch, R. G. (Eds.), Boca Raton, FL: Taylor & Francis Group

Joda, A. E., Tayo, F.and Aina, B. A. (2018) 'Quality assessment of ciprofloxacin tablets obtained from community pharmacies in Lagos, Nigeria', *Ife Journal of Science*, 20 (1)

John Wiley and Sons (1986) *International dictionary of medicine and biology,* New York, N. Y: John Wiley & Sons

Johnson, A. P. (2015) 'Surveillance of antibiotic resistance', *Philosophical Transactions of the Royal Society B: Biological Sciences*, 370, 20140080

Johnston, S. A. and May, R. C. (2010) 'The human fungal pathogen *Cryptococcus neoformans* escapes macrophages by a phagosome emptying mechanism that is inhibited by Arp2/3 complex-mediated actin polymerisation', *PLoS Pathogens,* 6, e1001041

Jonas, D., Walev, I., Berger, T., Liebetrau, M., Palmer, M. and Bhakdi. S. (1994) 'Novel path to apoptosis: small transmembrane pores created by staphylococcal α-toxin in T lymphocytes evoke internucleosomal DNA degradation', *Infection and Immunity*, 62, 1304–1312

Jonsson, K., Signas, C., Muller, H. P. and Lindberg, M. (1991) 'Two different genes encode fibronectin binding proteins in *Staphylococcus aureus*; the complete nucleotide sequence

and characterization of the second gene', *European Journal of Biochemistry*, 202, 1041-1048

Josse, J., Laurent, F. and Diot, A. (2017) 'Staphylococcal Adhesion and Host Cell Invasion: Fibronectin-Binding and Other Mechanisms', *Frontiers in Microbiology*, 8, 2433.

Josse, J., Velard, F. and Gangloff, S. C. (2015) '*Staphylococcus aureus* vs. osteoblast: relationship and consequences in osteomyelitis', *Frontiers in Cellular and Infection Microbiology*, 5, 85

Julien, C., Marcouiller, F., Bretteville, A., El Khoury, N. B., Baillargeon, J., Hébert, S. S., and Planel, E. (2012) 'Dimethylsulfoxide induces both direct and indirect tau hyperphosphorylation', *PLoS One* **7,** e40020

Jurisic, V. and Bumbasirevic, V. (2008) '*In vitro* assays for cell death determination', *Archive of Oncology*, 16, 49-54

Kahl, B. C., Becker, K. and Löffler, B. (2016) 'Clinical significance and pathogenesis of staphylococcal small colony variants in persistent infections', *Clinical Microbiology Reviews*, 29, 401–427

Kahl, B. C., Goulian, M., Van Wamel, W., Herrmann, M., Simon, S. M., Kaplan, G., Peters, G. and Cheung, A. L. (2000) '*Staphylococcus aureus* RN6390 replicates and induces apoptosis in a pulmonary epithelial cell line', *Infection and Immunity*, 68, 5385–5392

Kali, A. (2015) 'Antibiotics and bioactive natural products in treatment of methicillin resistant *Staphylococcus aureus*: A brief review', *Pharmacognosy Reviews*, 9, 29–34

Kalinowski, D. S. and Richardson, D. R. (2005) 'The evolution of iron chelators for the treatment of iron overload disease and cancer', *Pharmacological Reviews*, 57, 547–583

Kamaruzzaman, N. F., Firdessa, R. and Good, L. (2016) 'Bactericidal effects of polyhexamethylene biguanide against intracellular *Staphylococcus aureus* EMRSA-15 and USA 300, *Journal of Antimicrobial Chemotherapy*, 71, 1252–1259

Kanazawa, A., Ikeda, T. and Endo, T. J. (1993) 'Polymeric phosphonium salts as a novel class of cationic biocides. II. Effects of counter anion and molecular weight on antibacterial activity of polymeric phosphonium salts', *Journal of Polymer Science Part A: Polymer Chemistry*, 31, 1441-1447

Kaneko, J. and Kamio, Y. (2004) 'Bacterial two-component and heteroheptameric pore-forming cytolytic toxins: structures, pore-forming mechanism, and organization of the genes', *Bioscience, Biotechnology, and Biochemistry*, 68, 981–1003

Kang, C. and Song, J. (2013) 'Antimicrobial resistance in Asia: current epidemiology and clinical implications', *Infection and Chemotherapy*, 45, 22-31

Karauzum, H. and Datta, S. K. (2017) 'Adaptive immunity against *Staphylococcus aureus'*, *Current Topics in Microbiology and Immunology*, 409, 419–439

Karchmer, A. W. (1991) '*Staphylococcus aureus* and vancomycin: the sequel', *Annals of Internal Medicine,* 115, 739-741

Kasela, M. and Malm, A. (2018) 'Overview of phenotypic methods used for differentiation of *Staphylococcus aureus*', *Current Issues in Pharmacy and Medical* Sciences, 31, 117-121

Katayama, Y., Ito, T. and Hiramatsu, K. (2000) 'A new class of genetic element, *Staphylococcus* cassette chromosome *mec*, encodes methicillin resistance in *Staphylococcus aureus'*, *Antimicrobial Agents and Chemotherapy*, 44, 1549–1555

Kaur, D. C. and Chate, S. S. (2015) 'Study of antibiotic resistance pattern in methicillin resistant *Staphylococcus aureus* with special reference to newer antibiotic', *Journal of Global Infectious Diseases,* 7, 78–84

Kavanaugh, J. S. and Horswill, A. R. (2016) 'Impact of environmental cues on staphylococcal quorum sensing and biofilm development', *The Journal of Biological Chemistry*, 291, 12556–12564

Kechrid, A., Perez-Vazquez, M., Smaoui, H., Hariga, D., Rodrıguez- Banos, M., Vindel, A., Baquero, F., Canton, R. and Del Campo, R. (2011) 'Molecular analysis of community-acquired methicillin-susceptible and resistant *Staphylococcus aureus* isolates recovered from bacteraemic and osteomyelitis infections in children from Tunisia', *Clinical Microbiology and Infection*, 17, 1020–1026

Kellner, R. (2000) 'Proteomics. Concepts and perspectives', *Fresenius Journal of Analytical Chemistry,* 366, 517-524

Kenawy, el-R., Worley, S. D. and Broughton, R. (2007) 'The chemistry and applications of antimicrobial polymers: a state-of-the-art review', *Biomacromolecules,* 8, 1359-1384

Kepp, O., Galluzzi, L., Lipinski, M., Yuan, J. and Kroemer, G. (2011) 'Cell death assays for drug discovery', *Nature Reviews Drug Discovery,* 10, 221-237

Khan, S. A. K., Shetty, P. J., Sarayu, L. Y, Chidambaram, A. and Ranganathan, R. (2012) 'Detection of *mec*A genes of methicillin-resistant *Staphylococcus aureus* by polymerase chain reaction', *International Journal of Health and Rehabilitation Sciences,* 1, 64-68

Kiedrowski, M. R., Kavanaugh, J. S., Malone, C. L., Mootz, J. M., Voyich, J. M., Smeltzer, M. S., Bayles, K. W. & Horswill, A. R. (2011) 'Nuclease modulates biofilm formation in community-associated methicillin-resistant *Staphylococcus aureus*', *PLoS ONE,* 6, e26714

Kim, C., Milheirico, C., Gardete, S., Holmes, M. A., Holden, M. T., de Lencastre, H. and Tomasz, A. (2012) 'Properties of a novel PBP2A protein homolog from *Staphylococcus aureus* strain

LGA251 and its contribution to the beta-lactam-resistant phenotype', *Journal of Biological Chemistry*', 287, 36854–36863

Kim, C.M. and Shin, S. H. (2009) 'Effect of iron-chelator deferiprone on the *in vitro* growth of staphylococci', *Journal of Korean Medical Science,* 24, 289 –295

Kim, S. H., Song, J. H., Chung, D. R., Thamlikitkul, V., Yang, Y., Wang, H., Lu, M., So, T. M., Hsueh, P. R., Yasin, R. M., Carlos, C. C., Pham, H. V., Lalitha, M. K., Shimono, N., Perera, J., Shibl, A. M., Baek, J. Y., Kang, C. I., Ko, K. S., Peck, K. R.; ANSORP Study Group (2012) 'Changing trends in antimicrobial resistance and serotypes of *Streptococcus pneumoniae* isolates in Asian Countries: an Asian network for surveillance of resistant pathogens (ANSORP) Study', *Antimicrobial Agents and Chemotherapy,* 56, 1418-1426

Kimanga, A. N. (2012) 'A situational analysis of antimicrobial drug resistance in Africa: are we losing the battle?', *Ethiopian Journal of Health Sciences,* 22, 135-143

Kintarak, S., Whawell, S. A., Speight, P. M., Packer, S. and Nair, S. P. (2004) 'Internalization of *Staphylococcus aureus* by human keratinocytes', *Infection and Immunity,* 72, 5668–5675

Kintarak, S., Whawell, S. A., Speight, P. M., Packer, S. and Nair, S. P. (2004) 'Internalization of *Staphylococcus aureus* by human keratinocytes', *Infection and Immunity,* 72, 5668–5675

Klein, E., Smith, D. L. and Laxminarayan, R. (2007) 'Hospitalizations and deaths caused by methicillin-resistant *Staphylococcus aureus,* United States, 1999–2005', *Emerging Infectious Diseases,* 13, 1840–1846

Klevens, R. M., Morrison, M. A., Fridkin, S. K., Reingold, A., Petit, S., Gershman, K., Ray, R., Harrison, L. H., Lynfield, R., Dumyati, G., Townes, J. M., Craig, A. S., Fosheim, G., McDougal, L. K. and Tenover, F. C. for the Active Bacterial Core Surveillance of the Emerging Infections Program Network1 (2006) 'community-associated methicillin-resistant *Staphylococcus aureus* and healthcare risk factors', *Emerging Infectious Diseases,* 12, 1991–1993

Kluytmans, J., van B. A. and Verbrugh, H. (1997) 'Nasal carriage of *Staphylococcus aureus*: epidemiology, underlying mechanisms, and associated risks', *Clinical Microbiology Reviews,* 10, 505-520

Knight, G. M., Budd, E. L., Whitney, L., Thornley, A., Al-Ghusein, H., Planche, T. and Lindsay, J, A. (2012) 'Shift in dominant hospital-associated methicillin-resistant *Staphylococcus aureus* (HA-MRSA) clones over time', *Journal of Antimicrobial Chemotherapy,* 67, 2514-2522

Knowles, D. P., Kappmeyer, L. S., Haney, D., Herndon, D. R., Fry, L. M., Munro, J. B., Sears, K., Ueti, M. W., Wise, L. N., Silva, M., Schneider, D. A., Grause, J., White. S. N., Tretina, K., Bishop, R. P., Odongo, D. O., Pelzel-McCluskey, A. M., Scoles, G. A., Mealey, R. H. and Silva, J. C. (2018) 'Discovery of a novel species, *Theileria haneyi* n. sp., infective to equids,

highlights exceptional genomic diversity within the genus *Theileria*: implications for apicomplexan parasite surveillance', *International Journal for Parasitology*, 48, 679–690

Ko, K. S., Lee, J., Suh, J. Y., Oh, W. S., Peck, K. R., Lee, N. Y.and Song, J. (2005) 'Distribution of Major Genotypes among Methicillin-Resistant *Staphylococcus aureus* Clones in Asian Countries', *Journal of Clinical Microbiology*, 43, 421–426

Kobayashi, N., Alam, M. M. and Urasawa, S. (2001) 'Genomic rearrangement of the *mec* regulator region mediated by insertion of IS*431* in methicillin-resistant staphylococci', *Antimicrobial Agents and Chemotherapy*, 45, 335–338

Kobayashi, N., Urasawa, S., Uehara, N. and Watanabe, N. (1999) 'Distribution of insertion sequence-like element IS*1272* and its position relative to methicillin resistance genes in clinically important *Staphylococci*', *Antimicrobial Agents and Chemotherapy*, 43, 2780–2782

Kock, R., Becker, K., Cookson, B., van Gemert-Pijnen, J. E., Harbarth, S., Kluytmans, J., Mielke, M., Peters, G., Skov, R. L., Struelens, M. J., Tacconelli, E., Navarro Torné, A., Witte, W. and Friedrich, A. W. (2010) 'Methicillin-resistant *Staphylococcus aureus* (MRSA): burden of disease and control challenges in Europe', *Euro Surveillance*, 15, 19688

Kolmer, J. A. (1924) 'Infection', In: *Infection, immunity and biologic therapy*, W. B. Philadelphia, Pa: Saunders Co., pp. 58–83

Kong, C., Neoh, H., and Nathan, S. (2016) 'Targeting *Staphylococcus aureus* toxins: a potential form of anti-virulence therapy', *Toxins*, 8, 72 (A)

Kong, E. F., Johnson, J. K. and Jabra-Rizk, M. A. (2016) 'Community-associated methicillin-resistant *Staphylococcus aureus*: an Enemy amidst Us', *PLoS Pathogens*, 12: e1005837

Kontra, J. M. (2012) 'Reduction of surgical site infections by pre-operative eradication of the *Staphylococcus aureus* carrier state: a paradigm whose time has come', *The Journal of Lancaster General Hospital*, 7, 1

Kosowska-Shick, K., McGhee, P. L. and Appelbaumm P. C. (2010) 'Affinity of ceftaroline and other β-lactams for penicillin-binding proteins from *Staphylococcus aureus* and *Streptococcus pneumoniae*', Antimicrob. Agents Chemother, 54, 1670–1677

Kot, B., Sytykiewicz, H. and Sprawka, I. (2018) 'Expression of the biofilm-associated genes in Methicillin-Resistant *Staphylococcus aureus* in biofilm and planktonic conditions', *International Journal of Molecular Sciences*, 19, 3487

Koziel, J., Maciag-Gudowska, A., Mikolajczyk, T., Bzowska, M., Sturdevant, D. E., Whitney, A. R., Shaw, L. N., DeLeo, F. R. and Potempa, J. (2009) 'Phagocytosis of S*taphylococcus aureus* by macrophages exerts cytoprotective effects manifested by the upregulation of antiapoptotic factors', *PloS One*, 4, e5210.

Kreienbuehl, L., Charbonney, E. and Eggimann, P. (2011) 'Community-acquired necrotizing pneumonia due to methicillin-sensitive *Staphylococcus aureus* secreting Panton-Valentine leukocidin: a review of case reports', *Annals of Intensive Care*, 1, 52

Kriegeskorte, A., König, S., Sander, G., Pirkl, A., Mahabir, E., Proctor, R. A., von Eiff, C., Peters, G. and Becker, K. (2011) 'Small colony variants of *Staphylococcus aureus* reveal distinct protein profiles', *Proteomics*, 11, 2476-2490

Kroemer, G., Marino, G. and Levine, B. (2010) 'Autophagy and the integrated stress response', *Molecular Cell*, 40, 280–293

Krut, O., Utermohlen, O., Schlossherr, X. and Kronke, M. (2003) 'Strain-specific association of cytotoxic activity and virulence of clinical *Staphylococcus aureus* isolates', *Infection and Immunity*, 71, 2716-2723

Kubica, M., Guzik, K., Koziel, J., Zarebski, M., Richter, W., Gajkowska, B., Golda, A., Maciag-Gudowska, A., Brix, K., Shaw, L., Foster, T. and Potempa, J. (2008) 'A potential new pathway for *Staphylococcus aureus* dissemination: the silent survival of *S. aureus* phagocytosed by human monocyte-derived macrophages', *PLoS ONE*, 3, e1409

Kullar, R., Davis, S. L., Levine, D. P. and Rybak, M. J. (2011) 'Impact of vancomycin exposure on outcomes in patients with methicillin-resistant *Staphylococcus aureus* bacteremia: support for consensus guidelines suggested targets', *Clinical Infectious Diseases*, 52, 975-981

Kumar, A. and Kumar, A. (2015) 'Role of *Staphylococcus aureus* virulence factors in inducing inflammation and vascular permeability in a mouse model of bacterial endophthalmitis', *PloS One*, 10, e0128423

Kumar, C. C. (1998) 'Signaling by integrin receptors', *Oncogene*, 17, 1365–1373

Kumar, D., Bansal, G., Narang, A., Basak, T., Abbas, T. and Dash, D. (2016) 'Integrating transcriptome and proteome profiling: Strategies and applications', *Proteomics*, 16, 2533-2544

Kumar, M., Singh, R., Meena, A., Patidar, B. S., Prasad, R., Chhabra, S. K. and Bansal, S. K. (2017) 'An improved 2-dimensional gel electrophoresis method for resolving human erythrocyte membrane proteins', *Proteomics Insights*, 8, 1178641817700880

Kumar, N., David, M. Z., Boyle-Vavra, S., Sieth, J. and Daum, R. S. (2015) 'High *Staphylococcus aureus* colonization prevalence among patients with skin and soft tissue infections and controls in an urban emergency department', *Journal of Clinical Microbiology*, 53, 810–815

Kuo, S. C., Chiang, M. C., Lee, W. S., Chen, L. Y., Wu, H. S., Yu, K. W., Fung, C. P. and Wang, F. D. (2012) 'Comparison of microbiological and clinical characteristics based on SCC*mec* typing in patients with community-onset methicillin-resistant *Staphylococcus aureus* (MRSA) bacteraemia', *International Journal of Antimicrobial Agents*, 39, 22–26

Kwiatkowska, B., Maslinska, M., Przygodzka, M., Dmowska-Chalaba, J., Dabrowska, J. and Sikorska-Siudek, K. (2013) 'Immune system as a new therapeutic target for antibiotics', *Advances in Bioscience and Biotechnology*, 4, 91-101

Laabei, M., Recker, M., Rudkin, J. K., Aldeljawi, M., Gulay, Z., Sloan, T. J., Williams, P., Endres, J. L., Bayles, K. W., Fey, P. D., Yajjala, V. K., Widhelm, T., Hawkins, E., Lewis, K., Parfett, S., Scowen, L., Peacock, S. J., Holden, M., Wilson, D., Read, T. D., van den Elsen, J., Priest, N. K., Feil, E. J., Hurst, L. D., Josefsson, E. and Massey, R. C. (2014) 'Predicting the virulence of MRSA from its genome sequence', *Genome Research*. 24, 839–849

Lacoma, A., Cano, V., Moranta, D., Regueiro, V., Domínguez-Villanueva, D., Laabei, M., M., González-Nicolau, V., Prat, A. C. and Bengoechea, J. A. (2017) 'Investigating intracellular persistence of *Staphylococcus aureus* within a murine alveolar macrophage cell line', *Virulence*, 8, 1761–1775

Lakhundi, S. and Zhang, K. (2018) 'Methicillin-Resistant *Staphylococcus aureus*: molecular characterization, evolution, and epidemiology, *Clinical Microbiology Reviews,* 31, e00020-18.

Lamikanra, A., Paul, B. D., Akinwole, O. B. and Paul, M. O. (1985) 'Nasal carriage of *Staphylococcus aureus* in a population of healthy Nigerian students', *The Pathological Society of Great Britain and Ireland*, 19, 211-216

Lapaille, M., Thiry, M., Perez, E., González-Halphen, D., Remacle, C. and Cardol, P. (2010) 'Loss of mitochondrial ATP synthase subunit beta (Atp2) alters mitochondrial and chloroplastic function and morphology in *Chlamydomonas'*, *Biochimica et Biophysica Acta*, 1797, 1533–1539

Laxminarayan, R., Duse, A., Wattal, C., Zaidi, A. K., Wertheim, H. F., Sumpradit, N., Vlieghe, E., Hara, G. L., Gould, I. M., Goossens, H., Greko, C., So, A. D., Bigdeli, M., Tomson, G., Woodhouse, W., Ombaka, E., Peralta, A. Q., Qamar, F. N., Mir, F., Kariuki, S., Bhutta, Z. A., Coates, A., Bergstrom, R., Wright, G. D., Brown, E. D. and Cars, O. (2013) 'Antibiotic resistance-the need for global solutions', *The Lancet Infectious Diseases Commission*, 13, 1057-1098

Le Moing, V., Alla, F., Doco-Lecompte, T., Delahaye, F., Piroth, L., Chirouze, C., Tattevin, P., Lavigne, J. P., Erpelding, M. L., Hoen, B., Vandenesch, F., Duval, X., VIRSTA study group (2015) '*Staphylococcus aureus* bloodstream infection and endocarditis – a prospective cohort study', *PLoS One,* 10:e0127385

Le, K. Y. and Otto, M. (2015) 'Quorum-sensing regulation in staphylococci-an overview', *Frontiers in Microbiology*, 6, 1174

Lee, B. Y., Singh, A., David, M. Z., Bartsch, S. M., Slayton, R. B., Huang, S. S., Zimmer, S. M., Potter, M. A., Macal, C. M., Lauderdale, D. S., Miller, L. G. and Daum, R. S. (2013) 'The economic burden of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA)', *Clinical Microbiology and Infection,* 19, 528-536

Lekshmi, A., Varadarajan, S. N., Lupitha, S. S., Indira, D., Mathew, K. A., Chandrasekharan Nair, A., Nair, M., Prasad, T., Sekar, H., Gopalakrishnan, A. K., Murali, A. and Santhoshkumar, T. R. (2017) 'A quantitative real-time approach for discriminating apoptosis and necrosis', *Cell Death Discovery*, 3, 16101

Levine, B. and Kroemer, G. (2008) 'Autophagy in the pathogenesis of disease', *Cell*, 132, 27–42

Levine, D. P., Fromm, B. S. and Reddy, B. R. (1991) 'Slow response to vancomycin or vancomycin plus rifampin in methicillin-resistant *Staphylococcus aureus* endocarditis', *Annals of Internal Medicine*, 115, 674-680

Levinson, W. and Jawetz, E. (2002*) Medical microbiology and immunology: examination and board review*, 7th edn., McGraw-Hill, London

Li, J., Olaleye, E., Kong, X., Zhou, T., Ma, Y., Jurach, J., Al Rugaie, O., Hider, R. C., Zhang, G., Alsam, S. and Abbate, V. (2016) 'Macromolecular iron-chelators via RAFT-polymerization for the inhibition of methicillin-resistant *Staphylococcus aureus* growth', *Polymer*, 87, 64-72

Li, M., Diep, B. A., Villaruz, A. E., Braughton, K. R., Jiang, X., DeLeo, F. R., Chambers, H. F., Lu, Y. and Otto, M. (2009) 'Evolution of virulence in epidemic community-associated methicillin-resistant *Staphylococcus aureus*', *Proceedings of the National Academy of Sciences of the United States of America*, 106, 5883-5888

Li, M., Du, X., Villaruz, A. E., Diep, B. A., Wang, D., Song, Y., Tian, Y., Hu, J., Yu, F., Lu, Y. and Otto, M. (2012) 'MRSA epidemic linked to a quickly spreading colonization and virulence determinant', *Nature Medicine*, 18, 816–819

Li, S., Skov, R. L., Han, X., Larsen, A. R., Larsen, J., Sørum, M., Wulf, M., Voss, A., Hiramatsu, K. and Ito, T. (2011) 'Novel types of staphylococcal cassette chromosome *mec* elements identified in clonal complex 398 methicillin-resistant *Staphylococcus aureus strains*', *Antimicrobial Agents and Chemotherapy*, 55, 3046–3050

Lim, D. and Strynadka, N. C. (2002) 'Structural basis for the beta lactam resistance of PBP2a from methicillin-resistant *Staphylococcus aureus*', *Nature Structural and Molecular Biology*, 9, 870–876

Limoli, D. H., Jones, C. J. and Wozniak, D. J. (2015) 'Bacterial extracellular polysaccharides in biofilm formation and function', *Microbiology Spectrum*, 3, 10

Lin, H., Li, L., Jia, X., Ward, D. M., and Kaplan, J. (2011) 'Genetic and biochemical analysis of high iron toxicity in yeast: Iron toxicity is due to the accumulation of cytosolic iron and occurs under both aerobic and anaerobic conditions', *The Journal of Biological Chemistry*, 286, 3851–3862

Lin, J., Yan, X. J., Zheng, L., Ma, H. H. and Chen, H. M. (2005) 'Cytotoxicity and apoptosis induction of some selected marine bacteria metabolites', *Journal of Applied Microbiology*, 99, 1373–1382

Lin, T., Lin, L. and Zhang, F. (2014) 'Review on molecular typing methods of pathogens', *Open Journal of Medical Microbiology*, 4, 147-152

Lin, Y. C. and Peterson, M. L. (2010) 'New insights into the prevention of staphylococcal infections and toxic shock syndrome', *Expert Review of Clinical Pharmacology*, 3, 753-757

Lina, G., Durand, G., Berchich, C., Short, B., Meugnier, H., Vandenesch, F., Etienne, J. and Enright, M. C. (2006) 'Staphylococcal chromosome cassette evolution in *Staphylococcus aureus* inferred from *ccr* gene complex sequence typing analysis', *Clinical Microbiology and Infection*, 12, 1175–1184

Lister, J. L. and Horswill, A. R. (2014) '*Staphylococcus aureus* biofilms: recent developments in biofilm dispersal', *Frontiers in Cellular and Infection Microbiology*, 4, 178

Liu, C., Bayer, A., Cosgrove, S. E., Daum, R. S., Fridkin, S. K., Gorwitz, R. J., Kaplan, S. L., Karchmer, A. W., Levine, D. P., Murray, B. E. J., Rybak, M., Talan, D. A. and Chambers, H. F. (2011) 'Clinical practice guidelines by the infectious diseases society of America for the treatment of methicillin resistant *Staphylococcus aureus* infections in adults and children', *Clinical Infectious Diseases*, 52, e18–55.

Liu, Q., Yeo, W. S. and Bae, T. (2016) 'The SaeRS two-component system of *Staphylococcus aureus*', *Genes*, 7, 81

Liu, Z. D. and Hider, R. C. (2002) 'Design of iron chelators with therapeutic application', *Coordination Chemistry Reviews*, 232, 151–171

Livermore, D. M. (2005) 'Tigecycline: what is it, and where should it be used?', *Journal of Antimicrobial Chemotherapy*, 56, 611-614

Löffler, B. H., Kahl, B. C., Grundmeier, M.. Strangfeld, K., Wagner, B., Fischer, U., Cheung, A. L., Peters, G., Osthoff, K. S. and Sinha, B. (2005) 'Multiple virulence factors are required for *Staphylococcus aureus*-induced apoptosis In endothelial cells', *Cellular Microbiology*, **7,** 1087–1097

Loffler, B., Hussain, M., Grundmeier, M., Bruck, M., Holzinger, D., Varga, G., Roth, J., Kahl, B. C., Proctor, R. A. and Peters, G. (2010) '*Staphylococcus aureus* Panton-Valentine leukocidin is a very potent cytotoxic factor for human neutrophils', *PLoS Pathogens*, 6(1)

Löffler, B., Tuchscherr, L., Niemann, S. and Peters, G. (2014) '*Staphylococcus aureus* persistence in non-professional phagocytes', *International Journal of Medical Microbiology*, 304, 170-176

Loncaric, I., Kubber-Heiss, A., Posautz, A., Stalder, G. L., Hoffmann, D., Rosengarten, R. and Walzer, C. (2013) 'Characterization of methicillin-resistant *Staphylococcus* spp. carrying the *mecC* gene, isolated from wildlife', Journal of Antimicrobial Chemotherapy, 68, 2222–2225

Lopez, J. L. (2007) 'Two-dimensional electrophoresis in proteome expression analysis', *Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences*, 849, 190–202

Los, M., Wesselborg, S. and Schulze-Osthoff, K. (1999) 'The role of caspases in development, immunity, and apoptotic signal transduction: lessons from knockout mice', *Immunity*, 10, 629-639

Low, D. E. and Nadler, H. L. (1997) 'A review of *in-vitro* antibacterial activity of quinupristin/dalfopristin against methicillin-susceptible and –resistant *Staphylococcus aureus*', *Journal of Antimicrobial Chemotherapy*, 39, Suppl A: 53–58

Lowy, F. D. (1998) '*Staphylococcus aureus* infections', *New England Journal of Medicine*, 339, 520–532

Lowy, F. D. (2003) 'Antimicrobial resistance: The example of *Staphylococcus aureus*', *Journal of Clinical Investigation*, 111, 1265–1273

Luo, G., Spellberg, B., Gebremariam, T., Lee, H., Xiong, Y. Q., French, S. W., Bayer, A. and Ibrahim, A. S. (2014) 'Combination therapy with iron chelation and vancomycin in treating murine staphylococcemia', *European journal of clinical microbiology and infectious diseases*, 33, 845–851

Luong, T. T. and Lee, C. Y. (2002) 'Overproduction of type 8 capsular polysaccharide augments *Staphylococcus aureus* virulence', *Infection and Immunity*, 70, 3389–3395

Luqman, A., Ebner, P., Reichert, S., Sass, P., Kabagema-Bilan, C., Heilmann, C., Ruth, P. and Götz, F. (2019) 'A new host cell internalisation pathway for SadA-expressing staphylococci triggered by excreted neurochemicals', *Cellular Microbiology*, 21:e13044

Luqman, A., Nega, M., Nguyen, M. T., Ebner, P. and Götz, F. (2018) 'SadA-expressing staphylococci in the human gut show increased cell adherence and internalization', *Cell Reports*, 22, 535–545

Ma, X. X., Ito, T., Chongtrakool, P. and Hiramatsu, K. (2006) 'Predominance of clones carrying Panton-Valentine leukocidin genes among methicillin-resistant *Staphylococcus aureus* strains isolated in Japanese hospitals from 1979-1985', *Journal of Clinical Microbiology*, 44, 4515-4527

Macey, M. G. (2007) *Flowcytometry, principle and applications*, 3rd ed., Humana Press, Totowa, pp. 1-31

Machado, P.R. L., Araújo, M. I. A. S., Carvalho, L., and Carvalho, E. M. (2004) 'Mecanismos de resposta imune às infecções', *Anais Brasileiros de Dermatologia*, 79, 647-662

Magdeldin, S., Enany, S., Yoshida, Y., Xu, B., Zhang, Y., Zureena, Z., Lokamani, I., Yaoita, E. and Yamamoto, T. (2014) 'Basics and recent advances of two dimensional- polyacrylamide gel electrophoresis', Clinical Proteomics, 11, 16

Mandell, G. L., Bennett, J. E. and Dolin, R. (2009) '*Mandell, Douglas, and Bennett's Principles and Practice of Infectious Diseases*', 7th edition, Philadelphia: Elsevier

Manzoni, C., Kia, D. A., Vandrovcova, J., Hardy, J., Wood, N. W., Lewis, P. A. and Ferrari, R. (2016) 'Genome, transcriptome and proteome: the rise of omics data and their integration in biomedical sciences', *Briefings in Bioinformatics*, 19, 286–302

Maree, C. L., Daum, R. S., Boyle-Vavra, S., Matayoshi, K. and Miller L. G. (2007) 'Community-associated methicillin-resistant *Staphylococcus aureus* isolates causing healthcare-associated infections', Emerg Infect Dis, 13, 236–242

Marek, A., Pyzik, E., Stępień-Pyśniak, D., Urban-Chmiel, R. and Jarosz, Ł. S. (2018) 'Association between the methicillin resistance of *Staphylococcus aureus* isolated from slaughter poultry, their toxin gene profiles and prophage patterns', *Current Microbiology*, 75, 1256–1266

Markowitz, N., Quinn, E. L. and Saravolatz, L. D. (1992) 'Trimethoprim-sulfamethoxazole compared with vancomycin for the treatment of *Staphylococcus aureus* infection', *Annals of Internal Medicine,* 117, 390–398

Marrack, P. and Kappler, J. (1990) 'The staphylococcal enterotoxins and their relatives', *Science*, 240, 705–711

Marx, J. J. (2002) 'Iron and infection: competition between host and microbes for a precious element', *Best Practice and Research: Clinical Haematology*, 15, 411-426

Masiuk, H., Kopron, K., Grumann, D., Goerke, C., Kolata, J., Jursa-Kulesza, J., Giedrys-Kalemba, S., Broker, B. M. and Holfreter, S. (2010) 'Association of recurrent furunculosis with Panton-Valentine leukocidin and the genetic background of *Staphylococcus aureus*, *Journal of Clinical Microbiology,* 48, 1527-1535

Massey, R. C., Kantzanou, M. N., Fowler, T., Day, N. P., Schofield, K., Wann, E. R., Berendt, A. R., Höök, M. and Peacock, S. J. (2001) 'Fibronectin-binding protein A of *Staphylococcus aureus* has multiple, substituting, binding regions that mediate adherence to fibronectin and invasion of endothelial cells', *Cellular Microbiology*, 3, 839–851

Matuschek, E., Brown, D. F. J. and Kahlmeter, G. (2014) Development of the EUCAST disk diffusion antimicrobial susceptibility testing method and its implementation in routine microbiology laboratories', *Clinical Microbiology and Infection*, 20, 255– 266

Maurer, M. H., and Kuschinsky, W. (2007) 'Proteomics', pp 737–769, in *Handbook of Neurochemistry and Molecular Neurobiology*: *Brain Energetics. Integration of Cellular and Molecular Processes*, Lajtha, A., Gibson, G. E., and Dienel, G. A., 3rd Edn., Springer, New York

McAdam, P. R., Templeton, K. E., Edwards, G. F., Holden, M. T., Feil, E. J., Aanensen, D. M., Bargawi, H. J., Spratt, B. G., Bentley, S. D., Parkhill, J., Enright, M. C., Holmes, A., Girvan, E. K., Godfrey, P. A., Feldgarden, M., Kearns, A. M., Rambaut, A., Robinson, D. A. and Fitzgerald, J. R. (2012) 'Molecular tracing of the emergence, adaptation and transmission of hospital-associated methicillin-resistant *Staphylococcus aureus*', *Proceedings of the National Academy of Sciences USA*, 109, 9107-9112

McAdow, M., Kim, H. K., DeDent, A. C., Hendrickx, A. P. A., Schneewind, O., and Missiakas, D. M. (2011) 'Preventing *Staphylococcus aureus* sepsis through the inhibition of its agglutination in blood', *PLoS Pathogens,* **7**, e1002307

McCall, L. I., Siqueira-Neto, J. L. and McKerrow, J. H. (2016) 'Location, location, location: five facts about tissue tropism and pathogenesis', *PLoS Pathogens,* 12, e1005519.

McClure, J., Conly, J. M., Lau, V., Elsayed, S., Louie, T., Hutchins, W. and Zhang, K. (2006) 'Novel Multiplex PCR Assay for Detection of the Staphylococcal Virulence Marker Panton-Valentine Leukocidin Genes and Simultaneous Discrimination of Methicillin-Susceptible from -Resistant Staphylococci', *Journal Of Clinical Microbiology*, 44, 1141–1144

McClure-Warnier, J. A., Conly, J. M. and Zhang, K. (2013) 'Multiplex PCR assay for typing of staphylococcal cassette chromosome *mec* types I to V in methicillin-resistant *Staphylococcus aureus*', *Journal of Visualized Experiments*, 5, 79

McCormick, J. K., Yarwood, J. M. and Schlievert, M. (2001) 'Toxic shock syndrome and bacterial superantigens: an update', *Annual Review of Microbiology*, 55, 77–104

McCormick, J., Peterson, M. L. and Schlievert, P. M. (2006) 'Toxins and superantigens of group A streptococci', p 47–58. *In* Fischetti, V. A., Novick, R. P., Ferretti, J. J., Portnoy, D. A. and Rood, J. I. (ed), *Gram-Positive Pathogens*, Washington DC: ASM Press

McDevitt, D., Francois, P., Vaudaux, P., and Foster, T. J. (1994) 'Molecular characterization of the clumping factor (fibrinogen receptor) of *Staphylococcus aureus*', *Molecular Microbiology*, 11, 237–248

McGavin, M. H., Krajewska-Pietrasik, D., Ryden, C. and Hook, M. (1993) 'Identification of a *Staphylococcus aureus* extracellular matrix-binding protein with broad specificity', *Infection and Immunity*, 61, 2479- 2485

Mehndiratta, P. L. and Bhalla, P. (2012) 'Typing of methicillin resistant *Staphylococcus aureus*: a technical review', *Indian Journal of Medical Microbiology*, 30, 16-23

Mellinghoff, S. C., Vehreschild, J. J., Liss, B. J. and Cornely, O. A. (2018)' Epidemiology of surgical site infections with *Staphylococcus aureus* in Europe: protocol for a retrospective, multicenter study', *Journal of Medical Internet Research Research Protocols*, 7, e63

Mempel, M., Schmidt, T., Weidinger, S., Schnopp, C., Foster, T., Ring, J. and Abeck, D. (1998) 'Role of *Staphylococcus aureus* surface-associated proteins in the attachment to cultured

HaCaT keratinocytes in a new adhesion assay', *Journal of Investigative Dermatology*, 111, 452–456

Mendes, R. E., Tsakris, A., Sader, H. S., Jones, R. N., Biek, D., McGhee, P., Appelbaum, P. C. and Kosowska-Shick, K. (2012) 'Characterization of methicillin-resistant *Staphylococcus aureus* displaying increased MICs of ceftaroline', *Journal of Antimicrobial Chemotherapy*, 67, 1321–1324

Menegotto, F., González-Cabrero, S., Cubero, Á., Cuervo, W., Muñoz, M., Gutiérrez, M. P., Simarro, M., Bratos, M. A. and Orduña, A. (2012) 'clonal nature and diversity of resistance, toxins and adhesins genes of methicillin-resistant *Staphylococcus aureus* collected in a Spanish hospital, *Infection, Genetics and Evolution,*12, 1751-1758

Menestrina, G., Dalla Serra, M., Comai, M., Coraiola, M., Viero, G., Werner, S., Colin, D. A., Monteil, H. and Prévost. G. (2003) 'Ion channels and bacterial infection: the case of beta-barrel pore-forming protein toxins of *Staphylococcus aureus*', *FEBS Letters*, 552, 54-60

Menzies, B. E. and Kourteva, I. (1998) 'Internalization of *Staphylococcus aureus* by endothelial cells induces apoptosis', *American Society for Microbiology*, 66, 5994–5998

Mermel, L. A., Cartony, J. M., Covington, P., Maxey, G. and Morse, D. (2011) 'Methicillin-resistant *Staphylococcus aureus* colonization at different body sites: a prospective, quantitative analysis', *Journal of Clinical Microbiology*, 49, 1119–1121

Méry, B., Guy, J. B., Vallard, A., Espenel, S., Ardail, D., Rodriguez-Lafrasse, C. and Rancoule CMagné, N. (2017) 'In Vitro cell death determination for drug discovery: a landscape review of real issues', *Journal of Cell Death*, 10, 1179670717691251.

Mesri, M. (2014) 'Advances in proteomic technologies and its contribution to the field of cancer', *Advances in Medicine*, 238045.

Méthot, P. O. and Alizon, S. (2014) 'What is a pathogen? Toward a process view of host-parasite interactions', *Virulence,* 5, 775-785

Meyer, E. A. (1974) 'How microorganisms cause disease', In: *Microorganisms and human disease*, New York, N.Y: Appleton-Century-Crofts, pp. 53–57

Michalik, S., Depke, M., Murr, A., Salazar, M. G., Kusebauch, U., Sun, Z., Meyer, T. C., Surmann, K., Pförtner, H., Hildebrandt, P., Weiss, S., Medina, L. M. P., Gutjahr, M., Hammer, E., Becher, D., Pribyl, T., Hammerschmidt, S., Deutsch, E. W., Bader, S. L., Hecker, M., Moritz, R. L., Mäder, U., Völker, U. and Schmidt, F. (2017) 'A global *Staphylococcus aureus* proteome resource applied to the *in vivo* characterization of host-pathogen interactions', *Scientific Reports*, 7, 9718

Milheirico, C., Oliveira, D. C. and de Lencastre (2007) 'Multiplex PCR strategy for subtyping the staphylococcal cassette chromosome *mec* Type IV in methicillin-resistant *Staphylococcus aureus*: SCC*mec* IV multiplex', *Journal of Antimicrobial Chemotherapy*, 60, 42-48

Millar, B. C., Loughrey, A., Elborn, J. S. and Moore, J. E. (2007) 'Proposed definitions of community-associated meticillin-resistant *Staphylococcus aureus* (CA-MRSA)', J Hosp Infect, 67, 109-113

Miller, L. G. and Diep, B. A. (2008) 'Clinical practice: colonisation, fomites, and virulence: rethinking the pathogenesis of community-associated methicillin-resistant *Staphylococcus aureus* infection', *Clinical Infectious Diseases,* 46, 752–760

Miller, W. R., Bayer, A. S. and Arias, C. A. (2016) 'Mechanism of action and resistance to daptomycin in *Staphylococcus aureus* and enterococci', *Cold Spring Harbor Perspectives in Medicine,* 6, a025361.

Miquel, S., Lagrafeuille, R., Souweine, B. and Forestier, C. (2016) 'Anti-biofilm activity as a health issue', *Frontiers in Microbiology,* 7, 592

Miragaia, M. (2018) 'Factors contributing to the evolution of mecA-mediated β-lactam resistance in staphylococci: update and new insights from Whole Genome Sequencing (WGS)', *Frontiers in Microbiology,* 9, 2723

Mitchell, G., Chen, C. and Portnoy, D. A. (2016) 'Strategies used by bacteria to grow in macrophages', *Microbiology Spectrum,* 4, 10

Moellering, R. C. (2008) 'Current treatment options for community-acquired methicillin-resistant *Staphylococcus aureus* infection', *Clinical Infectious Diseases*, 46, 1032–1037

Moellering, R. C. (2011) 'MRSA: the first half century', *Journal of Antimicrobial Chemotherapy*, doi:10.1093/jac/dkr437

Mohr, J. F. and Murray, B. E. (2007) 'Point: vancomycin is not obsolete for the treatment of infection caused by methicillin-resistant *Staphylococcus aureus*', *Clinical Infectious Diseases*, 44, 1536–1542

Moisan, H., Pruneau, M. and Malouin, F. (2010) 'Binding of ceftaroline to penicillin-binding proteins of *Staphylococcus aureus* and *Streptococcus pneumoniae',* J. Antimicrob. Chemother, 65, 713–716

Monecke, S., Slickers, P., Gawlik, D., Müller, E., Reissig, A., Ruppelt-Lorz, A., Akpaka, P. E., Bandt, D., Bes, M., Boswihi, S. S., Coleman, D. C., Coombs, G. W., Dorneanu, O. S., Gostev, V. V., Ip, M., Jamil, B., Jatzwauk, L., Narvaez, M., Roberts, R., Senok, A., Shore, A. C., Sidorenko, S. V., Skakni, L., Somily, A. M., Syed, M. A., Thürmer. A., Udo. E. E., Vremeră, T., Zurita, J. and Ehricht, R. (2018) 'Molecular typing of ST239-MRSA-III from diverse geographic locations and the evolution of the SCCmec III element during its intercontinental spread', *Frontiers in Microbiology,* 9, 1436

Monecke, S., Jatzwauk, L., Müller, E., Nitschke, H., Pfohl, K., Slickers, P., Reissig, A., Ruppelt-Lorz, A. and Ehricht, R. (2016) 'Diversity of SCC*mec* elements in *Staphylococcus aureus* as observed in South-Eastern Germany', *PLoS One,* 11, e0162654

Monecke, S., Müller, E., Dorneanu, O. S., Vremeră, T. and Ehricht, R. (2014) 'Molecular typing of MRSA and of clinical *Staphylococcus aureus* isolates from Iaşi, Romania', *PLoS One,* 9, e97833

Monecke, S., Coombs, G., Shore, A.C., Coleman, D.C., Akpaka, P., Borg, M., Chow, H., Ip, M., Jatzwauk, L. Jonas, D., Kadlec, K., Kearns, A., Laurent, F., O'Brien, F. G., Pearson, J., Ruppelt, A., Schwarz, S,, Scicluna, E., Slickers, P., Tan, H. L., Weber, S. and Ehricht, R. (2011) 'A field guide to pandemic, epidemic and sporadic clones of methicillin-resistant *Staphylococcus aureus*', *PloS ONE,* 6(4)

Montgomery, C. P., Boyle-Vavra, S. and Daum, R. S. (2010) 'Importance of the global regulators Agr and SaeRS in the pathogenesis of CA-MRSA USA300 infection', *PLoS One,* 5, e15177

Moodley, A., Oosthuysen, W. F., Dusé, A. G., Marais, E.; South African MRSA Surveillance Group (2010) 'Molecular characterization of clinical methicillin-resistant *Staphylococcus aureus* isolates in South Africa', *Journal of Clinical Microbiology,* 48, 4608-4611

Moore, C. L., Osaki-Kiyan, P., Perri, M., Donabedian, S., Haque, N. Z., Chen, A. and Zervos, M. J. (2010) 'USA600 (ST45) Methicillin-resistant *Staphylococcus aureus* bloodstream infections in urban Detroit', *Journal of Clinical Microbiology*, 48, 2307-2310

Morar, M.**,** Bhullar, K.**,** Hughes, D. W.**,** Junop, M. and Wright, G. D**. (**2009) 'Structure and mechanism of the lincosamide antibiotic adenylyltransferase LinB', *Structure* 17, 1649–1659

Morell, E. A. and Balkin, D. M. (2010) 'Methicillin-resistant *Staphylococcus aureus*: a pervasive pathogen highlights the need for new antimicrobial development', *Yale Journal of Biology and Medicine,* 83, 223-233

Morgan, D. J., Okeke, I. N., Laxminarayan, R., Perencevich, E. N. and Weisenberg, S. (2011) 'Non-prescription antimicrobial use worldwide: a systematic review', *The Lancet. Infectious Diseases*, 11, 692–701

Morlock, B. A., Spero, L. and Johnson, A, D. (1980) 'Mitogenic activity of staphylococcal exfoliative toxin', *Infection and Immunity*, 30, 381–384

Morrison, J. M., Anderson, K. L., Beenken, K. E., Smeltzer, M. S. and Dunman, P. M. (2012) 'The staphylococcal accessory regulator, SarA, is an RNA-binding protein that modulates the mRNA turnover properties of late-exponential and stationary phase Staphylococcus aureus cells', *Frontiers in Cellular and Infection Microbiology*, 2, 26

Morrison, M. A., Hageman, J. C. and Klevens, R.M. (2006) 'Case definition for community-associated methicillin-resistant *Staphylococcus aureus*', *Journal of Hospital Infections*, 62(241)

Murthy, A., De Angelis, G., Pittet, D., Schrenzel, J., Uckay, I. and Harbarth, S. (2010) 'Cost-effectiveness of universal MRSA screening on admission to surgery', Clinical Microbiology and Infection, 16, 1747–1753

Musser, J. M., Schlievert, P. M., Chow, A. W., Ewan, P., Kreiswirth, B. N., Rosdahl, V. T., Naidu, A. S., Witte, W. and Selander, R. K. (1990) 'A single clone of *Staphylococcus aureus* causes the majority of cases of toxic shock syndrome', *Proceedings of the National Academy of Science U S A*, 87, 225–229

Naber, C. K. (2009) '*Staphylococcus aureus* bacteremia: epidemiology, pathophysiology, and management strategies', *Clinical Infectious Diseases*, 48, 231–237

Naimi, T. S., LeDell, K. H., Como-Sabetti, K., Borchandt, S. M., Boxrud, D. J., Etienne, J., Johnson, S. K., Vabdenesch, F., Fridkin, S., O'Boyle, C., Danila, R. N. and Lynfield, R. (2003) 'Comparison of community and health care-associated methicillin resistant *Staphylococcus aureus* infection', *The Journal of the American Medical Association*, 290, 2976-2984

Namvar, A. E., Khodaei, F., Bijari, A. and Lari, A. R. (2015) 'Detection of integrons and Staphylococcal Cassette Chromosome (SCC*mec*) types in *Staphylococcus aureus* isolated from burn and non-burn patients', *International Journal of Health Sciences (Qassim)*, 9, 440-445

Nanra, J. S., Buitrago, S. M., Crawford, S., Ng, J., Fink, P. S., Hawkins, J., Scully, I. L., McNeil, L. K., Aste-Amézaga, J. M., Cooper, D., Jansen, K. U. and Anderson, A. S. (2013) 'Capsular polysaccharides are an important immune evasion mechanism for *Staphylococcus aureus'*, *Human Vaccines and Immunotherapeutics*, 9, 480–487

Nasir, I. A., Babyo, A., Emeribe, A. U, Sani, N. O. (2015) 'Surveillance for antibiotic resistance in Nigeria: Challenges and possible solutions', *Trends in Medical Research*, 10, 106-113

Nathwani, D., Davey, P. G. and Marwick, C. A. (2010) 'MRSA: treating people with infection', BMJ Clinical Evidence, 0922

Negroni, A., Cucchiara, S. and Stronati, L. (2015) 'Apoptosis, necrosis, and necroptosis in the gut and intestinal homeostasis', *Mediators of Inflammation*, 250762

Neumann, Y., Bruns, S. A., Rohde, M., Prajsnar, T. K., Foster, S. J. and Schmitz, I. (2016) 'Intracellular *Staphylococcus aureus* eludes selective autophagy by activating a host cell kinase', *Autophagy*, 12, 2069–2084

Nguyen, H. M. and Graber, C. J. (2010) 'Limitations of antibiotic options for invasive infections caused by methicillin-resistant *Staphylococcus aureus*: is combination therapy the answer?', *Journal of Antimicrobial Chemotherapy*, 65, 24–36

Nickerson, E. K., Hongsuwan, M., Limmathurotsakul, D., Wuthiekanun, V., Shah, K. R., Srisomang, P., Mahavanakul, W., Wacharaprechasgul, T., Fowler, V. G., West, T. E.,

Teerawatanasuk, N., Becher, H., White, N. J., Chierakul, W., Day, N. P. and Peacock, S. J. (2009) '*Staphylococcus aureus* bacteraemia in a tropical setting: patient outcome and impact of antibiotic resistance', *PLoS One*, 4(1)

Nikbakht, M., Ahangarzadeh R. M., Hasani, A., Nahaei, M., Sadeghi, J. and Jedari S. S. (2017) 'Molecular characterization and antimicrobial susceptibility patterns of Methicillin-Resistant *Staphylococcus aureus* isolates in Tabriz, Northwest of Iran', *Archives of Pediatric Infectious Diseases*, 5, e61620

Niu, C., Yu, D., Wang, Y., Ren, H., Jin, Y., Zhou, W., Li, B., Cheng, Y., Yue, J., Gao, Z. and Liang, L. (2013) 'Common and pathogen-specific virulence factors are different in function and structure', *Virulence*, 4, 473–482

Noeske, J., Huang, J., Olivier, N. B., Giacobbe, R. A., Zambrowski, M., Catea, J. H. D. (2014) 'Synergy of streptogramin antibiotics occurs independently of their effects on translation', *Antimicrobial Agents and Chemotherapy*, 58, 5269–5279

Novick, R. P. and Jiang, D. (2003) 'The staphylococcal saeRS system coordinates environmental signals with agr quorum sensing', *Microbiology*, 149, 2709–2717

Novick, R. P., Ross, H. F., Projan, S. J., Kornblum, J., Kreiswirth, B. and Moghazeh, S. (1993) 'Synthesis of staphylococcal virulence factors is controlled by a regulatory RNA molecule', *The EMBO Journal*, 12, 3967-3975

Nübel, U., Roumagnac, P., Feldkamp, M., Song, J. H., Ko, K. S., Huang, Y. C., Coombs, G., Ip, M., Westh, H., Skov, R., Struelens, M. J., Goering, R. V., Strommenger, B., Weller, A., Witte, W. and Achtman, M. (2008) 'Frequent emergence and limited geographic dispersal of methicillin-resistant *Staphylococcus aureus*', *Proceedings of the National Academy of Sciences U. S. A.*, 105, 14130–14135

Nunez, G., London, L., Hockenbery, D., Alexander, M., McKearn, J. P. and Korsmeyer, S. J. (1990) 'Deregulated Bcl-2 gene expression selectively prolongs survival of growth factor-deprived hemopoietic cell lines', *Journal of Immunology*, 144, 3602–3610

Nuzzo, I., Sanges, M. R., Folgore, A. and Carratelli, C. R. (2000) 'Apoptosis of human keratinocytes after bacterial invasion', *FEMS Immunology and Medical Microbiology*, 27, 235–240

O'Riordan, K. and Lee, J. C. (2004) '*Staphylococcus aureus* capsular polysaccharides', *Clinical Microbiology Reviews*, 17, 218–234

O'Toole, R. D., Drew, W. L., Dahlgren, B. J. and Beaty, H. N. (1970) 'An outbreak of methicillin-resistant *Staphylococcus aureus* infection - observations in hospital and nursing home', *Journal of the American Medical Association*, 213, 257-263

Obidike, E. O., Anigbo, G., Igbodo, C. (2009) 'Sensitivity pattern of bacterial isolates in childhood sepsis in clinical practice at Onitsha', *Nigerian Journal of Clinical Practice*, 12, 302-305

O'Brien, L., Kerrigan, S. W., Kaw, G., Hogan, M., Penadés, J., Litt, D., Fitzgerald, D. J., Foster, T. J. and Cox, D. (2002) 'Multiple mechanisms for the activation of human platelet aggregation by *Staphylococcus aureus*: roles for the clumping factors *ClfA* and *ClfB*, the serine-aspartate repeat protein SdrE and protein A', *Molecular Microbiology*, 44, 1033-1044

OECD (2016) Safety assessment of transgenic organisms in the environment, vol 5: OECD consensus documents, harmonisation of regulatory oversight in biotechnology, OECD Publishing, Paris, http://dx.doi.org/10.1787/9789264253018-en

Office for national statistics-ONS (2013) Deaths involving MRSA: 2008 to 2012

Oie, S., Huang, Y., Kamiya, A., Konishi, H. and Nakazawa, T. (1996) 'Efficacy of disinfectants against biofilm cells of methicillin-resistant *Staphylococcus aureus*', *Microbios*, 85, 223–230

O'Keeffe, K. M., Wilk, M. M., Leech, J. M., Murphy, A. G., Laabei, M., Monk, I. R., Massey, R. C., Lindsay, J. A., Foster, T. J., Geoghegan, J. A. and McLoughlin, R. M. (2015) 'Manipulation of autophagy in phagocytes facilitates *Staphylococcus aureus* bloodstream infection, *Infection and Immunity*, 83, 3445-3457

Okeke, I. N. (2016) 'Laboratory systems as an antibacterial resistance containment tool in Africa', *African Journal of Laboratory Medicine*, 5, 497

Okeke, I. N., Lamikanra, A. and Edelman, R. (1999) 'Socioeconomic and behavioural factors leading to acquired bacterial resistance to antibiotics in developing countries', *Emerging Infectious Diseases*, 5, 18-27

Okon, K. O., Basset, P., Uba, A., Lin, J., Oyawoye, B., Shittu, A. O. and Blanc, D. S. (2009) 'Co-occurrence of predominant Panton-Valentine leukocidin-Positive Sequence Type (ST) 152 and Multidrug-Resistant ST 241 *Staphylococcus aureus* clones in Nigerian hospitals', *Journal of Clinical Microbiology*, 47, 3000-3003

Okon, K. O., Shittu, A. O., Kudi, A. A., Umar, H., Becker, K. and Schaumburg, F. (2014) 'Population dynamics of Staphylococcus aureus from Northeastern Nigeria in 2007 and 2012', *Epidemiology and Infection*, 142, 1737-1740

Okuma, K., Iwakawa, K., Turnidge, J. D., Grubb, W. B., Bell, J. M., O'Brien, F. G., Coombs, G. W., Pearman, J. W., Tenover, F. C., Kapi, M., Tiensasitorn, C., Ito, T. and Hiramatsu, K. (2002) 'Dissemination of new methicillin-resistant *Staphylococcus aureus* clones in the community', *Journal of Clinical Microbiology*, 40, 4289-4294

Olatunji, F. O., Fadeyi, A., Ayanniyi, A. A. and Akanbi, A. A. (2007) 'Non-gonococcal bacterial agents of conjunctivitis and their antibiotic susceptibility patterns in Ilorin, Nigeria', *African Journal of Medicine and Medical Sciences*, 36, 243-247

Oliveira, D. C., Tomasz, A. and de Lencastre, H. (2001) 'The evolution of pandemic clones of methicillin-resistant *Staphylococcus aureus*: identification of two ancestral genetic backgrounds and the associated mec elements', *Microbial Drug Resistance*, 7, 349–361

Olson, M. E. and Horswill, A. R. (2013) '*Staphylococcus aureus* osteomyelitis: bad to the bone', *Cell Host Microbe*, 13, 629–631

Olufunmiso, O., Tolulope, I., and Roger, C. (2017) 'Multidrug and vancomycin resistance among clinical isolates of *Staphylococcus aureus* from different teaching hospitals in Nigeria', *African Health Sciences*, 17, 797–807

Omar, N. Y., Ali, H. A. S., Harfoush, R. A. H. and El Khayat, E. H. (2014) 'Molecular typing of methicillin resistant *Staphylococcus aureus* clinical isolates on the basis of protein A and coagulase gene polymorphisms', *International Journal of Microbiology*, http://dx.doi.org/10.1155/2014/650328

Omuse, G., Van Zyl, K.N., Hoek, K., Abdulgader, S., Kariuki, S., Whitelaw, A. and Revathi, G. (2016) 'Molecular characterization of *Staphylococcus aureus* isolates from various healthcare institutions in Nairobi, Kenya: a cross sectional study', *Annals of Clinical Microbiology and Antimicrobials*, 15, 51

Onanuga, A. and Eboh, D. D. (2015) 'Biological Activities of Ciprofloxacin tablets and Gentamicin injections commonly used in Nigeria', *Journal of Pharmaceutical and Biomedical Sciences*, 14 (13)

Oreagba, I. A., Oshikoya, K. A., Ogar, C., Adefurin, A. O., Ibrahim, A., Awodele, O. and Oni, Y. (2017) 'Adverse reactions to fluoroquinolones in the Nigerian population: an audit of reports submitted to the National Pharmacovigilance Centre from 2004 to 2016', *Pharmacology Research and Perspectives*, 5, e00297

Osterholm, M. T., Hedberg, C. W. and Moore, K. A. (2000) 'Epidemiologic principles', In: Mandell, G. L., Bennett, J. E. and Dolin, R. (eds.) *Principles and practice of infectious diseases*, Philadelphia, Pa: Churchill Livingstone, pp. 156–167

Otero, L. H., Rojas-Altuve, A., Llarrull, L. I., Carrasco-López, C., Kumarasiri, M., et al. (2013) 'How allosteric control of *Staphylococcus aureus* penicillin binding protein 2a enables methicillin resistance and physiological function', PNAS, 110, 16808–16813

Otobo, U. N., Wala, P. G. and Agbagwa, O. E. (2018) 'Occurrence of Vancomycin-Resistant *Staphylococcus Aureus* (VRSA) in clinical and community isolates within the University of Port Harcourt', *Advances in Biotechnology and Microbiology*, 11, 555816

Otter, J. A. and French, G. L. (2012) 'Community-associated methicillin resistant *Staphylococcus aureus*: the case for a genotypic definition', *Journal of Hospital Infection*, 81, 143–148

Otter, J. A., Klein, J. L., Watts, T. L., Kearns, A. M. and French, G. L. (2007) 'Identification and control of an outbreak of ciprofloxacin-susceptible EMRSA-15 on a neonatal unit', J Hosp Infect, 67, 232-239

Otto, M. (2008) 'Staphylooccal biofilms', *Current Topics in Microbiology and Immunology*, 322, 207-228

Otto, M. (2012) 'Molecular basis of *Staphylococcus epidermidis* infections', *Seminars in Immunopathology*, 34, 201-214 (b)

Otto, M. (2012) 'MRSA virulence and spread', *Cellular Microbiology*, 14, 1513–1521

Otto, M. (2014) '*Staphylococcus aureus* toxins', *Current Opinion in Microbiology*, 0, 32-37

Owolabi, J. B. and Olorioke, R. C. (2015) 'Prevalence and antimicrobial susceptibility of methicillin-resistant *Staphylococcus aureus* and coagulase-negative Staphylococci isolated from apparently healthy university students in Ota, Nigeria, *Journal of Natural Sciences Research*, 5 (24)

Oyama, T., Miyazaki, M., Yoshimura, M., Takata, T., Ohjimi, H. and Jimi, S. (2016) 'Biofilm-forming Methicillin-Resistant *Staphylococcus aureus* survive in kupffer cells and exhibit high virulence in mice', *Toxins*, 8, 198

Özlem, S. A. (2017) '*In Vitro* cytotoxicity and cell viability assays: principles, advantages, and disadvantages', *Genotoxicity - A Predictable Risk to Our Actual World*, Larramendy, M. L. and Soloneski, S., IntechOpen

Pacifici, G. M. (2011) 'Pharmacokinetics of cephalosporins in the neonate: a review', *Clinics*, 66, 1267-1274

Paharik, A. E., and Horswill, A. R. (2016) 'The staphylococcal biofilm: adhesins, regulation, and host response', *Microbiology Spectrum*, 4, 16

Palenzuela, L., Hahn, N. M., Nelson, R. P. Jr., Arno, J. N., Schobert, C., Bethel, R., Ostrowski, L. A., Sharma, M. R., Datta, P. P., Agrawal, R. K., Schwartz, J. E. and Hirano, M. (2005) 'does linezolid cause lactic acidosis by inhibiting mitochondrial protein synthesis?', *Clinical Infectious Diseases*, 40, e113–116

Pantosti, A., Sanchini, A. and Monaco, M. (2007) 'Mechanisms of antibiotic resistance in *Staphylococcus aureus'*, *Future Microbiology*, 2007, 2, 323–334

Parija, S. C. (2009) *Textbook of Microbiology and Immunology*, India: Elsevier

Park, P. W., Rosenbloom, J., Abrams, W. R. and Mecham, R. P. (1996) 'Molecular Cloning and Expression of the Gene for Elastin-Binding Protein (ebpS) in *Staphylococcus aureus*', *Journal of Biological Chemistry*, 271, 15803-15809

Patel, J. B., Jevitt, L. A., Hageman, J., McDonald, L. C. and Tenover, F. C. (2006) 'An association between reduced susceptibility to daptomycin and reduced susceptibility to vancomycin in *Staphylococcus aureus*', *Clinical Infectious Disease*, 42, 1652–1653

Patti, J. M., Jonsson, H., Guss, B., Switalski, L. M., Wiberg, K., Lindberg, M. and Hook, M. (1992) 'Molecular characterization and expression of a gene encoding a *Staphylococcus aureus* collagen adhesin', *Journal of Biological Chemistry*, 267, 4766-4772

Pavlova, A., Parks, J. M., Oyelere, A. K. and Gumbart, J. C. (2017) 'Toward the rational design of macrolide antibiotics to combat resistance', *Chemical Biology and Drug Design*., 90, 641–652

Peacock, S. J. and Paterson, G. K. (2015) 'Mechanisms of Methicillin Resistance in *Staphylococcus aureus, Annual Review of Biochemistry*, 84, 577–601

Peacock, S. J., Moore, C. E., Justice, A., Kantzanou, M., Story, L., Mackie, K., O'Neill, G. and Day, N. P. (2002) 'Virulent combinations of adhesin and toxin genes in natural populations of *Staphylococcus aureus*', *Infection and Immunity*, 70, 4987–4996

Pechous, R. D. (2017) 'With friends like these: the complex role of neutrophils in the progression of severe pneumonia', *Frontiers in Cellular and Infection Microbiology*, 7, 160

Pérez-Llarena, F. J. and Bou, G. (2016) 'Proteomics as a Tool for studying bacterial virulence and antimicrobial resistance', *Frontiers in Microbiology*, 7, 410

Peterson, J. W. (1996) 'Bacterial Pathogenesis', In: *Medical Microbiology*, Baron, S., 4th Edn., Galveston (TX)

Pfaller, M. A., Flamm, R. K., Mendes, R. E., Streit, J. M., Smart, J. I., Hamed, K. A, Duncan, L. R. and Sader, H. S. (2019) 'Ceftobiprole activity against Gram-positive and -negative pathogens collected from the United States in 2006 and 2016', Antimicrob Agents Chemother, 63:e01566-18

Pham, T. D. M., Ziora, Z. M. and Blaskovich, M. A. T. (2019) 'Quinolone antibiotics', *Medchemcomm*, 10, 1719-1739

Pi, H. And Helmann, J. D. (2017) 'Sequential induction of Fur-regulated genes in response to iron limitation in *Bacillus subtilis'*, *Proceedings of the National Academy of Sciences of the United States of America*, 114, 12785–12790

Pihan, P., Carreras-Sureda, A. and Hetz, C. (2017) 'BCL-2 family: integrating stress responses at the ER to control cell demise', *Cell Death and Differentiation*, 24, 1478–1487

Pillai, S. K., Gold, H. S., Sakoulas, G., Wennersten, C., Moellering, R. C. Jr. and Eliopoulos, G. M. (2007) 'Daptomycin nonsusceptibility in *Staphylococcus aureus* with reduced vancomycin susceptibility is independent of alterations in MprF', *Antimicrobial Agents and Chemotherapy*, 51, 2223–2225

Planet, P. J., Larussa, S. J., Dana, A., Smith, H., Xu, A., Ryan, C., Uhlemann, A. C., Boundy, S., Goldberg, J., Narechania, A., Kulkarni, R., Ratner, A. J., Geoghegan, J. A., Kolokotronis, S. O. and Prince, A. (2013) 'Emergence of the epidemic methicillin-resistant *Staphylococcus aureus* strain USA300 coincides with horizontal transfer of the arginine catabolic mobile element and *speG*-mediated adaptations for survival on skin', *mBio*, 4(6)

Plata, K., Rosato, A. E. And Wegrzyn, G. (2009) 'Staphylococcus aureus as an infectious agent: overview o biochemistry and molecular genetics of its pathogenicity', Acta Biochimica Polonica, 56, 587-612

Popovich, K. J. and Weinstein, R. A. (2009) 'Commentary: the graying of methicillin-resistant *Staphylococcus aureus*', *Infection Control & Hospital Epidemiology*, 30, 9-12

Porto, J. P., Santos, R. O., Gontijo Filho, P. P. and Ribas, R. M. (2013) 'Active surveillance to determine the impact of methicillin resistance on mortality in patients with bacteremia and influences of the use of antibiotics on the development of MRSA infection', *Revista da Sociedade Brasileira de Medicina Tropical*, 46, 713-718

Postma, B., Poppelier, M.J., van Galen, J.C., Prossnitz, E.R., van Strijp, J.A., de Haas, C.J. and van Kessel, K.P. (2004) 'Chemotaxis inhibitory protein of *Staphylococcus aureus* binds specifically to the C5a and formylated peptide receptor', *Journal of Immunology*, 172, 6994–7001

Poulin, R. and Combes, C. (1999) 'The concept of virulence: interpretations and implications', *Parasitology Today*, 15, 474-475

Prasad, P., Sun, J., Danner, R. L. and Natanson, C. (2012) 'Excess deaths associated with tigecycline after approval based on noninferiority trials', *Clinical Infectious Diseases*, 54, 1699–1709

Prince, L. R., Graham, K. J., Connolly, J., Anwar, S., Ridley, R., Sabroe, I., Foster, S. J. and Whyte, M. K.. (2012) '*Staphylococcus aureus* induces eosinophil cell death mediated by α-hemolysin', *PLoS One*. 7, e31506

Proctor, R. A. (2008) 'Role of folate antagonists in the treatment of methicillin-resistant *Staphylococcus aureus* infection', *Clinical Infectious Diseases*, 46, 584–593

Proctor, R. A., Kriegeskorte, A., Kahl, B. C., Becker, K., Loffler, B. and Peters G. (2014) '*Staphylococcus aureus* small colony variants (SCVs): a road map for the metabolic pathways involved in persistent infections', *Frontiers in Cellular and Infection Microbiology*, 4, 99

Proctor, R. A., von Eiff, C., Kahl, B. C., Becker, K., McNamara, P., Herrmann, M. and Peters, G. (2006) 'Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections', *Nature Reviews Microbiology*, 4, 295-305

Public Health England-PHE (2016) Annual Epidemiological Commentary: Mandatory MRSA, MSSA and *E. coli* bacteraemia and *C. difficile* infection data, 2015/16, London: Public Health England

Qiu, D. H., Huang, Z. L., Zhou, T., Shen, C. and Hider, R.C. (2011) '*In vitro* inhibition of bacterial growth by iron chelators', *FEMS Microbiology Letters*, 314, 107–111

Qu, X., Zou, Z., Sun, Q., Luby-Phelps, K., Cheng, P., Hogan, R. N., Gilpin, C. and Levine, B. (2007) 'Autophagy gene-dependent clearance of apoptotic cells during embryonic development', Cell, 128, 931-946

Queck, S. Y., Khan, B. A., Wang, R., Bach, T. H., Kretschmer, D., Chen, L., Kreiswirth, B. N., Peschel, A., Deleo, F. R. andOtto, M. (2009) 'Mobile genetic element encoded cytolysin connects virulence to methicillin resistance in MRSA', *PLOS Pathogens*, 5(7)

Queipo-Ortuño, M. I., De Dios Colmenero, J., Macias, M., Bravo, M. J. and Morata, P. (2008) 'Preparation of bacterial DNA template by boiling and effect of immunoglobulin G as an inhibitor in real-time PCR for serum samples from patients with brucellosis', *Clinical and Vaccine Immunology*, 15, 293-296

Queval, C. J., Brosch, R. and Simeone, R. (2017) 'The macrophage: a disputed fortress in the battle against *Mycobacterium tuberculosis*', *Frontiers in Microbiology, 8*, 2284

Raad, I., Darouiche, R., Dupuis, J., Abi-Said, D., Gabrielli, A., Hachem, R., Wall, M., Harris, R., Jones, J., Buzaid, A., Robertson, C., Shenaq, S., Curling, P., Burke, T. and Ericsson, C. The Texas Medical Center Catheter Study Group (1997) 'Central venous catheter coated with minocycline and rifampin for the prevention of catheter –related colonization and bloodstream infections: a randomized, double –blind trial', *Annals of Internal Medicine*, 127, 267-274

Raad, I., Hachem, R., Hanna, H., Girgawy, E., Rolston, K., Whimbey, E., Husni, R. and Bodey, G. (2001) 'Treatment of vancomycin-resistant enterococcal infections in the immunocompromised host: quinupristin-dalfopristin in combination with minocycline', *Antimicrobial Agents and Chemotherapy*, 45, 3202-3204

Ranjbar, R., Karami, A., Farshad, S., Giammanco, G. M. and Mammina, C. (2014) 'Typing methods used in the molecular epidemiology of microbial pathogens', *New Microbiologica*, 37, 1-15

Rasigade, J. P., Laurent, F., Lina, G., Meugnier, H., Bes, M., Vandenesch, F., Etienne, J. and Tristan, A. (2010) 'Global distribution and evolution of Panton-Valentine leukocidin-positive methicillin-susceptible *Staphylococcus aureus*, 1981- 2007', *Journal of Infectious Diseases*, 201, 1589-1597

Rasschaert, G., Vanderhaeghen, W., Dewaele, I., Janez, N., Huijsdens, X., Butaye, P. and Heyndrickx, M. (2009) 'Comparison of fingerprinting methods for typing methicillin-

resistant *Staphylococcus aureus* sequence type 398', *Journal of Clinical Microbiology*, 47, 3313-3322

Rayner, C. and Munckhof, W. J. (2005) 'Antibiotics currently used in the treatment of infections caused by *Staphylococcus aureus*', *Internal Medicine Journal*, 35, S3-S16

Rebic, V., Budimir, A., Aljicevic, M., Bektas, S., Vranic, S. M. and Rebic, D. (2016) 'Typing of Methicillin Resistant *Staphylococcus Aureus* using DNA fingerprints by Pulsed-field Gel Electrophoresis', Acta Informatica Medica: AIM, 24, 248–252

Reddy, P. N., Srirama, K. and Dirisala, V. R. (2017) 'An update on clinical burden, diagnostic tools, and therapeutic options of *Staphylococcus aureus*', *Infectious Diseases: Research and Treatment*, 10, 1–15

Reichmann, N. T. and Pinho, M. G. (2017) 'Role of SCC*mec* type in resistance to the synergistic activity of oxacillin and cefoxitin in MRSA', *Scientific Reports*, 7, 6154

Ribet, D. and Cossart, P. (2015) 'How bacterial pathogens colonize their hosts and invade deeper tissues', *Microbes and Infection*, 17, 173-183

Ridley, R. A., Douglas, I. and Whawell, S. A. (2012) 'Differential adhesion and invasion by *Staphylococcus aureus* of epithelial cells derived from different anatomical sites', *Journal of Medical Microbiology*, 61, 1654-1661

Riss, T. L., Moravec, R. A., Niles, A. L., Duellman, S., Benink, H. A., Worzella, T. J. and Minor, L. (2013) 'Cell Viability Assays', in *Assay Guidance Manual* [Internet], Sittampalam, G. S., Coussens, N.P., Brimacombe, K., *et al*., editors, 2004 [Updated 2016 Jul 1], Bethesda (MD): Eli Lilly & Company and the National Center for Advancing Translational Sciences

Roberts, S. and Chambers, S. (2005) 'Diagnosis and management of *Staphylococcus aureus* infections of the skin and soft tissue', *Internal Medicine Journal*, 35, S97–S105

Robinson, D. A., Kearns, A. M., Holmes, A., Morrison, D., Grundmann, H., Edwards, G., O'Brien, F. G., Tenover, F. C., McDougal, L. K., Monk, A. B. and Enright, M. C. (2005) 'Re-emergence of early pandemic *Staphylococcus aureus* as a community-acquired methicillin-resistant clone', *Lancet*, 365, 1256–1258

Rodriguez, M., Hogan, P. G., Satola, S. W., Crispell, E., Wylie, T., Gao, H., Sodergren, E., Weinstock, G. M., Burnham, C. A. and Fritz, S. A. (2015) 'Discriminatory indices of typing methods for epidemiologic analysis of contemporary *Staphylococcus aureus* strains', *Medicine*, 94, e1534

Rodvold, K. A. and McConeghy, K. W. (2014) 'Methicillin-Resistant *Staphylococcus aureus* therapy: past, present, and future', *Clinical Infectious Diseases*, 58, S20–S27

Rollin, G., Tan, X., Tros, F., Dupuis, M., Nassif, X., Charbit, A. and Coureuil, M. (2017) 'Intracellular survival of *Staphylococcus aureus* in endothelial cells: a matter of growth or persistence', *Frontiers in Microbiology*, 8, 1354

Rong, D., Wu, Q., Xu, M., Zhang, J. and Yu, S. (2017) 'Prevalence, virulence genes, antimicrobial susceptibility, and genetic diversity of *Staphylococcus aureus* from retail aquatic products in China', *Frontiers in Microbiology*, 8, 714

Rooijakkers, S.H., and Van Strijp, J.A. (2007) 'Bacterial complement evasion', *Molecular Immunology*, 44, 23–32

Roomaney, R. A., Pillay-van Wyk, V., Awotiwon, O. F., Dhansay, A., Groenewald, P., Joubert, J. D., Nglazi, M. D., Nicol, E. and Bradshaw, D. (2016) **'**Epidemiology of lower respiratory infection and pneumonia in South Africa (1997-2015): a systematic review protocol', *BMJ Open*, 6, e012154

Roos, W. P., et al. (2016) 'DNA damage and the balance between survival and death in cancer biology', *Nature Reviews Cancer*, 16, 20–33

Rose, W. E. and Rybak, M. J. (2006) 'Tigecycline: first of a new class of antimicrobial agents', *Pharmacotherapy*, 26, 1099–1110

Rotimi, V. O., Orebanjo, O., Banjo, T. O., Nwobu, R. and Onyenefa, P. I. (1987) 'Occurrence and antibiotic susceptibility profile of methicillin-resistant *Staphylococcus aureus* in LUTH Lagos', *Central African Journal of Medicine*, 33, 95–98

Ruhe, J. J., Monson, T., Bradsher, R. W. and Menon, A. (2005) 'Use of long-acting tetracyclines for methicillin-resistant *Staphylococcus aureus* infections: Case Series and Review of the Literature', *Clinical Infectious Diseases*, 40, 1429–1434

Ruimy, R., Armand-Lefevre, L., Barbier, F., Ruppe, E., Cocojaru, R., Mesli, Y., Maiga, A., Benkalfat, M., Benchouk, S., Hassaine, H., Dufourcq, J. B., Nareth, C., Sarthou, J. L., Andremont, A. and Feil, E. J. (2009) Comparisons between geographically diverse samples of carried *Staphylococcus aureus*', *Journal of Bacteriology*, 191, 5577-5583

Ruimy, R., Maiga, A., Armand-Lefevre, L., Maiga, I., Diallo, A., Koumare, A. K., Ouattara, K., Soumare, S., Gaillard, K., Lucet, J. C., Andremont, A., Feil, E. J. (2008) 'The carriage population of *Staphylococcus aureus* from Mali is composed of a combination of pandemic clones and the divergent Panton-Valentine leukocidin-positive genotype ST152', *Journal of Bacteriology*, 190, 3962-3968

Ruppe, E., Ois Barbier, F., Mesli, Y., Maiga, A., Cojocaru, R., Benkhalfat, M., Benchouk, S., Hassaine, H., Maiga, I., Diallo, A., Koumare, A., Ouattara, K., Soumare, S., Dufourcq, J., Nareth, C., Sarthou, J., Andremont, A. and Ruimy, R. (2009) 'Diversity of staphylococcal cassette chromosome *mec* structures in methicillin-resistant *Staphylococcus epidermidis* and

Staphylococcus haemolyticus strains among outpatients from four countries', *Antimicrobial Agents and Chemotherapy*, 53, 442–449

Rutherford, S. T. and Bassler, B. L. (2012) 'Bacterial quorum sensing: its role in virulence and possibilities for its control', *Cold Spring Harbor Perspectives in Medicine*, 2, a012427

Ryu, S., Song, P. I., Seo, C. H., Cheong, H. and Park, Y. (2014) 'Colonization and infection of the skin by *S. aureus*: immune system evasion and the response to cationic antimicrobial peptides', *International Journal of Molecular Sciences*, 15, 8753–8772

Sabat, A. J., Budimir, A., Nashev, D., Sá-Leão, R., van Dijl J. M., Laurent, F., Grundmann, H., Friedrich, A. W.; ESCMID Study Group of Epidemiological Markers (ESGEM) (2013) 'Overview of molecular typing methods for outbreak detection and epidemiological surveillance', *Eurosurvellance*, 18(4)

Salgado-Pabon, W., Breshears, L., Spaulding, A. R., Merriman, J. A., Stach, C. S., Horswill, A. R., Peterson, M. L. and Schlieverta, P. M. (2013) 'Superantigens are critical for *Staphylococcus aureus* infective endocarditis, sepsis, and acute kidney injury', *MBio*, 4: e00494–13

Sanya, T. E., Titilayo, O. F., Adisa, R. and Segun, J. S. (2013) 'Use of antibiotics among non-medical students in a Nigerian university', *African Health Sciences*, 13, 1149–1155

Saravolatz, L. D. and Eliopoulos, G. M. (2003) 'Quinupristin-dalfopristin and linezolid: evidence and opinion', *Clinical Infectious Diseases*, 36, 473–481

Saravolatz, L. D., Pawlak, J. and Johnson, L. B. (2012) 'In vitro susceptibilities and molecular analysis of vancomycin-intermediate and vancomycin-resistant *Staphylococcus aureus* isolates', *Clinical Infectious Diseases*, 55, 582–586

Satoskar, A. A. and Nadasdy, T. (2017) *Bacterial infections and the kidney*, Springer International Publishing

Scanvic, A., Denic, L., Gaillon, S., Giry, P., Andremont, A. and Lucet, J. C.(2001) 'Duration of colonisation by methicillin-resistant *Staphylococcus aureus* after hospital discharge and risk factors for prolonged carriage', *Clinical Infectious Diseases*, 32, 1393–1398

Schaumburg, F., Alabi, A. S., Peters, G. and Becker, K. (2014) 'New epidemiology of *Staphylococcus aureus* infection from Africa', *Clinical Microbiology and Infection*', 20, 589-596

Schleucher, R. D., Gaessler, M. and Knobloch, J. (2008) 'Panton-Valentine leukocidin-producing methicillin-sensitive *Staphylococcus aureus* as a cause for recurrent, contagious skin infections in young, healthy travelers returned from a tropical country: a new worldwide public health problem?', *Journal of Travel Medicine*, 15, 137-139

Schlievert, P. M., Case, L. C., Strandberg, K. L., Tripp, T. J., Lin, Y. C. and Peterson, M. L. (2007) 'Vaginal *Staphylococcus aureus* superantigen profile shift from 1980 and 1981 to 2003, 2004, and 2005', *Journal of Clinical Microbiology*, 45, 2704–2707

Schmitz, F.J., Verhoef, J. and Fluit, A. C. (1999) 'Prevalence of resistance to MLS antibiotics in 20 European university hospitals participating in the European SENTRY surveillance programme', *Journal of Antimicrobial Chemotherapy*, 43, 783-792

Schnaith, A., Kashkar, H., Leggio, S. A., Addicks, K., Krönke, M. and Krut, O. (2007) '*Staphylococcus aureus* subvert autophagy for induction of caspase-independent host cell death', *Journal of Biological Chemistry*, 282, 2695-2706

Schriever, C. A., Fernández, C., Rodvold, K. A., Danziger, L. H. (2005) 'Daptomycin: a novel cyclic lipopeptide antimicrobial', *American Journal of Health-System Pharmacy*, 62, 1145–1158

Schwarz-Linek, U., Werner, J.M., Pickford, A.R., Gurusiddappa, S., Kim, J.H., Pilka, E.S., Briggs, J. A., Gough, T. S., Hook, M., Campbell, I. D. and Potts, J. R. (2003) 'Pathogenic bacteria attach to human fibronectin through a tandem beta-zipper', Nature, 423, 177–181

Schweichel, J. U. and Merker, H. J. (1973) 'The morphology of various types of cell death in prenatal tissues', *Teratology*, 7, 253–266

Serafini, A., Boldrinm, F., Palù, G. and Manganelli, R. (2009) 'Characterisation of a *Mycobacterium tuberculosis* ESX-3 conditional mutant: essentiality and rescue by iron and zinc', *Journal of Bacteriology*, 191, 6340–6344

Shachar, D. B., Kahana, N., Kampel, V., Warshawsky, A., Youdim, M. B. (2004) 'Neuroprotection by a novel brain permeable iron chelator, VK-28, against 6-hydroxydopamine lession in rats', *Neuropharmacology*, 46, 254-263

Shahbaz, K. (2017) 'Cephalosporins: pharmacology and chemistry', *Pharmaceutical and Biological Evaluations*, 4, 234-238

Shapiro-Ilan, D. I., Fuxa, J. R., Lacey, L. A., Onstad, D. W. and Kaya, H. K. (2005) 'Definitions of pathogenicity and virulence in invertebrate pathology', *Journal of Invertebrate Pathology*, 88, 1–7

Shariff, K. A., Richards, E. P. and Townes, J. M. (2013) 'Clinical management of septic arthritis', *Current Rheumatology Reports*, 15, 332

Sharma, M., Nunez-Garcia, J., Kearns, A. M., Doumith, M., Butaye, P. R., Argudín, M. A., Lahuerta-Marin, A., Pichon, B., AbuOun, M., Rogers, J., Ellis, R. J., Teale, C. and Anjum, M. F. (2016) 'Livestock-associated methicillin resistant *Staphylococcus aureus* (LA-MRSA) clonal complex (CC) 398 isolated from UK animals belong to European lineages', *Frontiers in Microbiology*, 7, 1741

Shaw, K. J. and Barbachyn, M. R. (2011) 'The oxazolidinones: past, present, and future', *Annals of the New York Academy of Sciences*, 1241, 48–70

Shet, A., Mathema, B., Mediavilla, J. R., Kishii, K., Mehandru, S., Jeane-Pierre, P., Laroche, M., Willey, B. M., Kreiswirth, N., Markowitz, M. and Kreiswirth, B. N. (2009) 'Colonization and subsequent skin and soft tissue infection due to Methicillin-Resistant *Staphylococcus aureus* in a cohort of otherwise healthy adults infected with HIV Type 1', *The Journal of Infectious Diseases*, 200, 88–93

Shiau, A. L., and Wu, C. L. (1998) 'The inhibitory effect of *Staphylococcus epidermidis* slime on the phagocytosis of murine peritoneal macrophages is interferon-independent', *Microbiology and Immunology*, 42, 33–40

Shinji, H., Yosizawa, Y., Tajima, A., Iwase, T., Sugimoto, S., Seki, K. and Mizunoe, Y. (2011) 'Role of fibronectin-binding proteins A and B in *in vitro* cellular infections and in vivo septic infections by Staphylococcus aureus', *Infection and Immunity*, 79, 2215-2223

Shittu, A. O., Lin, J. and Kolawole, D. O. (2006) 'Antimicrobial susceptibility patterns of *Staphylococcus aureus* and characterization of MRSA in South-Western Nigeria', *Wounds*, 18, 77-84

Shittu, A. O., Okon, K., Adesida, S., Oyedara, O., Witte, W., Strommenger, B., Layer, F. and Nübel, U. (2011) 'Antibiotic resistance and molecular epidemiology of *Staphylococcus aureus* in Nigeria', *BMC Microbiology*, 11(92)

Shittu, A., Oyedara, O., Abegunrin, F., Okon, K., Raji, A., Taiwo, S., Ogunsola, F., Onyedibe, K. and Elisha, G. (2012) 'Characterization of methicillin-susceptible and resistant staphylococci in the clinical setting: a multicentre study in Nigeria', *BMC Infectious Diseases*, 12(286)

Shompole, S., Henon, K. T., Liou, L. E., Dziewanowska, K., Bohach, G. A. and Bayles, K. W. (2003) 'Biphasic intracellular expression of *Staphylococcus aureus* virulence factors and evidence for *Agr*-mediated diffusion sensing', *Molecular Microbiology*, 49, 919-927

Shore, A. C. and Coleman, D. C. (2013) 'Staphylococcal cassette chromosome *mec*: Recent advances and new insights', *International Journal of Medical Microbiology*, 303, 350– 359

Shore, A. C., Brennan, O. M., Deasy, E. C., Rossney, A. S., Kinnevey, P. M., Ehricht, R., Monecke, S. and Coleman, D. C. (2012) 'DNA microarray profiling of a diverse collection of nosocomial methicillin-resistant *Staphylococcus aureus* isolates assigns the majority to the correct sequence type and staphylococcal cassette chromosome *mec* (SCC*mec*) type and results in the subsequent identification and characterization of novel SCC*mec*-SCCM1 composite islands', *Antimicrobial Agents and Chemotherapy*, 56, 5340-5355

Shorr, A. F. (2007) 'Epidemiology of staphylococcal resistance', *Clinical Infectious Diseases*, 45, S171–S176

Silverman, J. A., Mortin, L. I., VanPraagh, A. D. G., Li, T. and Alder, J. (2005) 'Inhibition of daptomycin by pulmonary surfactant: *In vitro* modeling and clinical impact', *Journal of Infectious Disease*, 191, 2149–2152

Single, A., Beetham, H., Telford, B. J., Guilford, P. and Chen, A. (2015) 'A comparison of real-time and endpoint cell viability assays for improved synthetic lethal drug validation', *Journal of Biomolecular Screening*, 20, 1286–1293

Sinha, B., François, P. P., Nüße, O., Foti, M., Hartford, O. M., Vaudaux, P., Foster, T. J., Lew, D. P., Herrmann, M. and Krause K.-H. (1999) 'Fibronectin-binding protein acts as *Staphylococcus aureus* invasin via fibronectin bridging to integrin a5β1', *Cellular Microbiology*, 1, 101–117

Skommer, J., Darzynkiewicz, Z. and Wlodkowic, D. (2010) 'Cell death goes LIVE: technological advances in real-time tracking of cell death', Cell Cycle, 9, 2330–2341

Skov, R., Christiansen, K., Dancer, S. J., Daum, R. S., Dryden, M., Huang, Y. C.and Lowy, F. D. (2012) 'Update on the prevention and control of community-acquired methicillin-resistant *Staphylococcus aureus* (CA-MRSA)', *International Journal of Antimicrobial Agents*, 39, 193–200

Slibinskas, R., Ražanskas, R., Zinkevičiūtė, R., and Čiplys, E. (2013) 'Comparison of first dimension IPG and NEPHGE techniques in two-dimensional gel electrophoresis experiment with cytosolic unfolded protein response in *Saccharomyces cerevisiae*', *Proteome Science*, 11, 36

Slover, C. M., Rodvold, K. A. and Danziger, L. H. (2007) 'Tigecycline: a novel broad-spectrum antimicrobial, *Annals of Pharmacotherapy*, 41, 965–972

Smith, L. M. and May, R. C. (2013) 'Mechanisms of microbial escape from phagocyte killing', *Biochemical Society Transactions*, 41, 475-490

Smith, T. L., Pearson, M. L., Wilcox, K. R., Cruz, C., Lancaster, M. V., Robinson-Dunn, B., Tenover, F. C., Zervos, M. J., Band, J. D., White, E. and Jarvis, W. R. (1999) 'Emergence of vancomycin resistance in *Staphylococcus aureus*. Glycopeptide-intermediate *Staphylococcus aureus* working group', *New England Journal of Medicine,* 340 493–501

Sollid, J. U. E., Furberg, A. S., Hanssen, A. M. and Johannessen, M. (2014) '*Staphylococcus aureus*: determinants of human carriage', *Infection, Genetics and Evolution*, 21, 531–541

Somerville, G. A. and Proctor, R. A. (2009) 'At the crossroads of bacterial metabolism and virulence factor synthesis in Staphylococci', *Microbiology and Molecular Biology reviews: MMBR*, 73, 233–248

Song, J. H., Hsueh, P. R., Chung, D. R., Ko, K. S., Kang, C. I., Peck, K. R.; ANSORP Study Group (2011) 'Spread of methicillin-resistant *Staphylococcus aureus* between the community and the hospitals in Asian countries: an ANSORP study', *Journal of Antimicrobial* Chemotherapy, 66, 1061-1069

Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J. E. (1996) 'Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore', *Science,* 274, 1859–1866

Song, L., Hobaugh, M. R., Shustak, C., Cheley, S., Bayley, H. and Gouaux, J. E. (1996) 'Structure of staphylococcal α-hemolysin, a heptameric transmembrane pore', *Science,* 274, 1859–1866

Song, M. D., Wachi, M., Doi, M., Ishino, F. and Matsuhashi, M. (1987) 'Evolution of an inducible penicillin-target protein in methicillin-resistant *Staphylococcus aureus* by gene fusion', *FEBS Letters,* 221, 167–171

South Central Antimicrobial Network (SCAN) (2018), 'Guidelines for Antibiotic Prescribing in the Community 2018', NHS North Hampshire Clinical Commissioning Group, http://www.nhsantibioticguidelines.org.uk

Sparling, P. F. (1983) 'Bacterial virulence and pathogenesis: an overview', *Reviews of infectious diseases,* 5, S637–S646

Spaulding, A. R., Salgado-Pabón, W.,Kohler, P. L., Horswill, A. R., Leung, D. Y. and Schlievert, P. M. (2013) 'Staphylococcal and streptococcal superantigen exotoxins', *Clinical Microbiology Reviews*, 26, 422-447

Spellberg, B., Hansen, G. R., Kar, A., Cordova, C. D., Price, L. B. and Johnson, J. R. (2016) 'Antibiotic resistance in humans and animals', Discussion Paper, National Academy of Medicine, Washington, DC

Speziale, P. and Geoghegan, J. A. (2015) 'Biofilm formation by staphylococci and streptococci: structural, functional, and regulatory aspects and implications for pathogenesis', *Frontiers in Cellular and Infection Microbiology*, 5, 31

Speziale, P., Pietrocola, G., Foster, T. J. and Geoghegan, J. A. (2014) 'Protein-based biofilm matrices in Staphylococci', *Frontiers in Cellular and Infection Microbiology*, 4, 171

Sritharan, M. (2006) 'Iron and bacterial virulence', *Indian Journal of Medical Microbiology*, 24, 163–164

Stach, C. S., Herrera, A. and Schlievert, P.M. (2014) 'Staphylococcal superantigens interact with multiple host receptors to cause serious diseases', *Immunologic Research*, 59, 177–181

Stefani, S., Chung, D. R., Lindsay, J. A., Friedrich, A. W., Kearns, A. M., Westh, H. and Mackenzie, F. M. (2012) 'Methicillin-resistant *Staphylococcus aureus* (MRSA): global

epidemiology and harmonisation of typing methods', *International Journal of Antimicrobial Agents*, 39, 273–82

Steinhaus, E. A. and Martignoni, M. E. (1970) *An Abridged Glossary of Terms Used in Invertebrate Pathology*, 2nd edition, USDA Forest Service, PNW Forest and Range Experiment Station

Strobel, M., Pförtner, H., Tuchscherr, L., Völker, U., Schmidt, F., Kramko, N., Schnittler, H. J., Fraunholz, M. J., Löffler, B., Peters, G. and Niemann, S. (2016) 'Post-invasion events after infection with *Staphylococcus aureus* are strongly dependent on both the host cell type and the infecting *S. aureus* strain', *Clinical Microbiology and Infection*, 22, 799-809

Strommenger, B., Braulke, C., Heuck, D., Schmidt, C., Pasemann, B., Nübel, U. and Witte, W. (2008) 'Spa Typing of *Staphylococcus aureus* as a Frontline Tool in Epidemiological Typing', *Journal of Clinical Microbiology*, 46, 574–581

Struck, S., Schmidt, U., Gruening, B., Jaeger, I. S., Hossbach, J., and Preissner, R. (2008) 'Toxicity versus potency: Elucidation of toxicity properties discriminating between toxins, drugs, and natural compounds', Genome Informatics, 20, 231-242

Struelens, M. J., Hawkey, P. M., French, G. L., Witte, W. and Tacconelli, E. (2009) 'Laboratory tools and strategies for methicillin-resistant *Staphylococcus aureus* screening, surveillance and typing: state of the art and unmet needs', *European Society of Clinical Microbiology and Infectious Diseases*, 15, 112-119

Stryjewski, M. E. and Corey, G. R. (2014) 'Methicillin-resistant *Staphylococcus aureus*: an evolving pathogen', *Clinical Infectious Diseases*, 58, S10–S19

Sturgill-Koszycki S., Schlesinger, P. H., Chakraborty, P., Haddix, P. L., Collins, H. L., Fok, A. K., Allen, R. D., Gluck, S. L., Heuser, J. and Russell, D. G. (1994) 'Lack of acidification in *Mycobacterium* phagosomes produced by exclusion of the vesicular proton-ATPase', *Science*, 263, 678–681

Suzuki, E., Kuwahara-Arai, K., Richardson, J. F. and Hiramatsu, K. (1993) 'Distribution of *mec* Regulator Genes in Methicillin-Resistant *Staphylococcus* Clinical Strains', *Antimicrobial Agents and Chemotherapy*, 37, 1219–1226

Taiwo, S. S. (2009) 'Methicillin resistance in *Staphylococcus aureus*: a review of the molecular epidemiology, clinical significance and laboratory detection methods', *West African Journal of Medicine*, 28, 281-290

Takada, Y., Ye, X. and Simon, S. (2007) 'The integrins', *Genome Biology*, 8(5)

Tanada, Y. and Fuxa, J.R. (1987) 'The host population', In: Fuxa, J. R. and Tanada, Y. (eds.), *Epizootiology of Insect Diseases*, Wiley, New York, NY, pp. 113–157

Tariq, A., Ali, H., Zafar, F., Sial, A. A., Hameed, K., Naveed, S., Shafiq, Y., Salim, S., Mallick, N. and Hasnain, H. (2017) 'A Systemic review on surgical site infections: classification, risk factors, treatment complexities, economical and clinical scenarios', *Journal of Bioequivalence and Bioavailability*, 9, 1

Tarrand, J. J., LaSala, P. R., Han, X.-Y., Rolston, K. V. and Kontoyiannis, D. P. (2012) 'Dimethyl sulfoxide enhances effectiveness of skin antiseptics and reduces contamination rates of blood cultures', *Journal of Clinical Microbiology*, 50, 1552–1557

Tattevin, P., Schwartz, B. S., Graber, C. J., Volinski, J., Bhukhen, A., Bhukhen, A., Mai, T. T., Vo, N. H., Dang, D. N., Phan, T. H., Basuino, L., Perdreau-Remington, F., Chambers, H. F. and Diep, B. A. (2012) 'Concurrent epidemics of skin and soft tissue infection and bloodstream infection due to community-associated methicillin-resistant *Staphylococcus aureus*', *Clinical Infectious Diseases*, 55, 781–788

Taubes, G. (2008) 'The bacteria fight back'. *Science*, 321, 356-361

Teng, T. S., Ji, A. L., Ji, X. Y., and Li, Y. Z. (2017) 'Neutrophils and immunity: from bactericidal action to being conquered', *Journal of Immunology Research*, 9671604

Tenover, F. C., Tickler, I. A., Goering, R. V., Kreiswirth, B. N., Mediavilla, J. R. and Persing, D. H. (2012) 'Characterization of nasal and blood culture isolates of methicillin-resistant *Staphylococcus aureus* from patients in United States Hospitals', *Antimicrobial Agents and Chemotherapy*, 56, 1324–1330

Tenson, T., Lovmar, M. and Ehrenberg, M. (2003) 'The mechanism of action of macrolides, lincosamides and streptogramin B reveals the nascent peptide exit path in the ribosome', *Journal of Molecular Biology*, 330, 1005–1014

Terico, A. T. and Gallagher, J. C. (2014) 'Beta-lactam hypersensitivity and cross-reactivity', *Journal of Pharmacy Practice*, 27, 530-544

Testoni, F., Montanaro, L., Poggi, A., Visai, L., Campoccia, D. and Arciola C. R. (2011) 'Internalization by osteoblasts of two *Staphylococcus aureus* clinical isolates differing in their adhesin gene pattern', *International Journal of Artificial Organs*, 34, 789–798

Thakker, M., Park, J. S., Carey, V. and Lee, J. C. (1998) '*Staphylococcus aureus* serotype 5 capsular polysaccharide is antiphagocytic and enhances bacterial virulence in a murine bacteremia model', *Infection and Immunity*, 66, 5183–5189

Thay, B., Wai, S. N. and Oscarsson, J. (2013) 'Staphylococcus aureus α-toxin-dependent induction of host cell death by membrane-derived vesicles', *PLoS One*, 8, e54661

Thomas, S. R. and Elkinton, J. S. (2004) 'Pathogenicity and virulence', *Journal of Invertebrate Pathology*, 85, 146–151

Thompson, M. G., Corey, B. W., Si, Y., Craft, D. W. and Zurawski, D. V. (2012) 'Antibacterial activities of iron chelators against common nosocomial pathogens', *Antimicrobial Agents and Chemotherapy*, 56, 5419-5421

Thomson, P. D. and Smith, D. J. (1994) 'What is infection?', *American Journal of Surgery*, 167, 7S–11S

Thwaites, G. E. (2010) 'The management of *Staphylococcus aureus* bacteremia in the United Kingdom and Vietnam: a multi-centre evaluation', *PLoS ONE*, 5(12)

Tiemersma, E. W., Bronzwaer, S. L., Lyytikäinen, O., Degener, J. E., Schrijnemakers, P., Bruinsma, N., Monen, J., Witte, W. and Grundman, H.; European Antimicrobial Resistance Surveillance System Participants (2004) 'Methicillin-resistant *Staphylococcus aureus* in Europe, 1999-2002', *Emerging Infectious Diseases*, 10, 1627-1634

Tong, S. Y., Davis, J. S., Eichenberger, E., Holland, T. L. and Fowler, V. G., Jr. (2015) '*Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management', *Clinical microbiology reviews* 28, 603–661

Torres, V. J., Attia, A. S., Mason, W. J., Hood, M. I., Corbin, B. D., Beasley, F. C., Anderson, K. L., Stauff, D. L., McDonald, W. H., Zimmerman, L. J., Friedman, D. B., Heinrichs, D. E., Dunman, P. M. and Skaar, E. P. (2010) '*Staphylococcus aureus* fur regulates the expression of virulence factors that contribute to the pathogenesis of pneumonia', *Infection and Immunity*, 78, 1618–1628

Toutain, P. L., Ferran, A. A., Bousquet-Melou, A., Pelligand, L. and Lees, P. (2016) 'Veterinary medicine needs new green antimicrobial drugs', *Frontiers in Microbiology*, 7, 1196

Tran, T. T., Munita, J. M. and Arias, C. A. (2015) 'Mechanisms of drug resistance: daptomycin resistance', *Annals of the New York Academy of Sciences*, 1354, 32-53

Traven, A. and Naderer, T. (2014) 'Microbial egress: a hitchhiker's guide to freedom', *PLoS Pathogens*, 10, e1004201

Truant, A. L. (2016) *Manual of commercial methods in clinical microbiology*, 2nd Edn., John Wiley and Sons Inc., Hoboken, New Jersey

Tsubakishita, S., Kuwahara-Arai, K., Baba, T. and Hiramatsu, K. (2010) 'Staphylococcal cassette chromosome mec-like element in *Macrococcus caseolyticus*', *Antimicrobial Agents and Chemotherapy*, 54, 1469–1475 (A)

Tsubakishita, S., Kuwahara-Arai, K., Sasaki, T. and Hiramatsu, K. (2010) 'Origin and molecular evolution of the determinant of methicillin resistance in staphylococci', *Antimicrobial Agents and Chemotherapy*, 54, 4352–4359

Tuchscherr, L., Medina, E., Hussain, M., Völker, W., Heitmann, V., Niemann, S., Holzinger, D., Roth, J., Proctor, R. A., Becker, K., Peters, G. and Löffler, B. (2011) '*Staphylococcus aureus*

phenotype switching: an effective bacterial strategy to escape host immune response and establish a chronic infection', *EMBO Molecular Medicine*, 3, 129–141

Tucker, K. A., Reilly, S. S., Leslie, C. S. and Hudson, M. C. (2000) 'Intracellular *Staphylococcus aureus* induces apoptosis in mouse osteoblasts', *FEMS Microbiology Letters*, 186, 151–156

Tuffs, S. W., James, D. B. A., Bestebroer, J., Richards, A. C., Goncheva, M. I., O'shea, M., Wee, B. A., Seo, K. S., Schlievert, P. M., Lengeling, A., van Strijp, J. A., Torres, V. J. and Fitzgerald, J. R. (2017) 'The *Staphylococcus aureus* superantigen SElX is a bifunctional toxin that inhibits neutrophil function', *PLoS Pathogens*, 13, e1006461

Turlej, A., Hryniewicz, W. and Empel, J. (2011) 'Staphylococcal cassette chromosome *mec* (*SCCmec*) classification and typing methods: an overview', *Polish Journal of Microbiology*', 60, 95-103

Tzianabos, A. O., Wang, J. Y. and Lee, J. C. (2001) 'Structural rationale for the modulation of abscess formation by *Staphylococcus aureus* capsular polysaccharides', *Proceedings of the National Academy of Sciences*, 98, 9365–9370

Ubani, U. A. (2009) 'Bacteriology of external ocular infections in Aba, South Eastern Nigeria', *Clinical and Experimental Optometry*, 92, 482-489

Uhlemann, A. C., Otto, M., Lowy, F. D. and DeLeo, F. R. (2014) 'Evolution of community- and healthcare-associated methicillin-resistant *Staphylococcus aureus*', *Infection, Genetics and Evolution*, 21, 563–574

Ullah, H. and Ali, S. (2017) 'Classification of antibacterial agents and their functions', pages 1-16, in: *Antibacterial Agents*, Ranjith Kumavath, IntechOpen, DOI: 10.5772/intechopen.68695,

Unachukwu, C. N., Obunge, O. K. and Odia, O. J. (2005) 'The bacteriology of diabetic foot ulcers in Port Harcourt, Nigeria', *Nigerian Journal of Medicine*, 14, 173–176

Urban, C. F., Lourido, S. and Zychlinsky, A. (2006) 'How do microbes evade neutrophil killing?', *Cellular Microbiology*, 8, 1687–1696

Uribe-Querol, E. and Rosales, C. (2017) 'Control of phagocytosis by microbial pathogens', *Frontiers in Immunology*, 8, 1368

Utsui, Y. and Yokota, T. (1985) 'Role of an altered penicillin-binding protein in methicillin- and cephem-resistant *Staphylococcus aureus*', *Antimicrobial Agents and Chemotherapy*, 28, 397-403

Valeva, A., Palmer, M. and Bhakdi, S. (1997) 'Staphylococcal α-toxin: formation of the heptameric pore is partially cooperative and proceeds through multiple intermediate stages', *Biochemistry*, 36, 13298–13304

van Belkum, A. (2016) ‘Hidden *Staphylococcus aureus* carriage: overrated or underappreciated?’, *MBio*, 7, 1–3

van Belkum, A., Verkaik, N. J., de Vogel, C. P., Boelens, H. A., Verveer, J., Nouwen, J. L., Verbrugh, H. A. and Wertheim, H. F. (2009) ‘Reclassification of *Staphylococcus aureus* nasal carriage types’, *Journal of Infectious Diseases*, 199, 1820-1826

van der Kooi-Pol, M. M., Sadaghian Sadabad, M., Duipmans, J. C., Sabat, A. J., Stobernack, T., Omansen, T. F., Westerhout-Pluister, G. N., Jonkman, M. F., Harmsen, H. J. and van Dijl, J. M. (2013) ‘Topography of distinct *Staphylococcus aureus* types in chronic wounds of patients with epidermolysis bullosa’, *PloS One*, 8, e67272

van Hal, S. J., Paterson, D. L. and Gosbell, I. B. (2011) ’Emergence of daptomycin resistance following vancomycin-unresponsive *Staphylococcus aureus* bacteraemia in a daptomycin-naive patient--a review of the literature’, *European Journal of Clinical Microbiology and Infectious Diseases*, 30, 603–610

Vandenesch, F., Naimi, T., Enright, M. C., Lina, G., Nimmo, G. R., Heffernan, H., Liassine, N., Bes, M., Greenland, T., Reverdy, M. E. and Etienne, J. (2003) ‘Community-acquired methicillin-resistant *Staphylococcus aureus* carrying Panton-Valentine leukocidin genes: worldwide emergence’, *Emerging Infectious Diseases*, 9, 978–984

Vann, J. M., and Proctor, R. A. (1988) ‘Cytotoxic effects of ingested *Staphylococcus aureus* on bovine endothelial cells: role of *S. aureus* α-hemolysin’, *Microbial Pathogenesis*, 4, 443–453

Vasileva, D., Janssen, H., Hönicke, D., Ehrenreich, A. and Bahl, H. (2012) ‘Effect of iron limitation and fur gene inactivation on the transcriptional profile of the strict anaerobe *Clostridium acetobutylicum*, *Microbiology*, 158, 1918–1929

Verdier, I., Durand, G., Bes, M., Taylor, K. L., Lina, G., Vandenesch, F., Fattom, A. I. and Etienne, J. (2007) ‘Identification of the capsular polysaccharides in *Staphylococcus aureus* clinical isolates by PCR and agglutination tests’, *Journal of Clinical Microbiology*, 45, 725-729

Vidlak, D. and Kielian, T. (2016) ‘Infectious dose dictates the host response during *S. aureus* orthopedic biofilm infection’, *Infection and Immunity*, doi:10.1128/IAI.00117-16

Villareal, V. A., Pilpa, R. M., Robson, S. A., Fadeev, E. A. and Clubb, R. T. (2008) ‘The IsdC protein from *Staphylococcus aureus* uses a flexible binding pocket to capture heme’, *Journal of Biological Chemistry*, 283, 31591-31600

Villegas-Estrada, A., Lee, M., Hesek, D., Vakulenko, S. and Mobashery, S. (2008) ‘Co-opting the cell wall in fighting methicillin-resistant *Staphylococcus aureus*: potent inhibition of PBP 2a by two anti-mrsa β-lactam antibiotics’, J Am Chem Soc. 130, 9212–9213

Vitale, I., , Manic, G., De Maria, R., Kroemer, G. and Galluzzi, L.. (2017) 'DNA damage in stem cells', *Molecular Cell*, 66, 306–319

Vivas, M. C. and Gutierrez, A. C. M. (2018) 'Typification methods and molecular epidemiology of *Staphylococcus aureus* with *Methicillin Resistance*, in *Staphylococcus aureus*, Hassan Hemeg, Hani Ozbak and Farhat Afrin, IntechOpen

von Eiff, C, Becker, K., Machka, K., Stammer, H. and Peters, G. (2001) 'Nasal carriage as a source of *Staphylococcus aureus* bacteremia', *New England Journal of Medicine,* 344, 11-16

von Eiff, C., Friedrich, A. W., Peters, G. and Becker, K. (2004) 'Prevalence of genes encoding for members of the staphylococcal leukotoxin family among clinical isolates of *Staphylococcus aureus*', *Diagnostic Microbiology and Infectious Disease,* 49, 157-162

von Graevenitz, A. (1977) 'The role of opportunistic bacteria in human disease', *Annual Review of Microbiology,* 31, 447–471

Vuong, C., Yeh, A. J., Cheung, G. Y. and Otto, M. (2016) 'Investigational drugs to treat methicillin-resistant *Staphylococcus aureus*', *Expert opinion on investigational drugs*, 25, 73–93

Wadhwani, T., Desai, K., Patel, D., Lawani, D., Bahaley, P., Joshi, P. and Kothar, V. (2008) 'Effect of various solvents on bacterial growth in context of determining MIC of various antimicrobials', Internet Scientific Publications, 7(1)

Walraven, C. J., North, M. S., Marr-Lyon, L., Deming, P., Sakoulas, G. and Mercier, R. C. (2011) 'Site of infection rather than vancomycin mic predicts vancomycin treatment failure in methicillin-resistant *Staphylococcus aureus* bacteremia', *Journal of Antimicrobial Chemotherapy*, 66, 2386–2392

Wang, B. and Muir, T. W. (2016) 'Regulation of virulence in *Staphylococcus aureus*: molecular mechanisms and remaining puzzles', *Cell Chemical Biology*, 23, 214–224

Wang, Y., Singh, R., Xiang, Y. and Czaja, M. J. (2010) 'Macroautophagy and chaperone-mediated autophagy are required for hepatocyte resistance to oxidant stress', Hepatology, 52, 266–277

Washburn, M. P. and Yates, J. R. 3rd (2000) 'Analysis of the microbial proteome', *Current Opinion in Microbiology,* 3, 292-297

Watkins, R. R., David, M. Z. and Salata, R. A. (2012) 'Current concepts on the virulence mechanisms of methicillin-resistant *Staphylococcus aureus*', *Journal of Medical Microbiology*, 61, 1179–1193

Weinstein, L. and Musher, D. M. (1969) 'Antibiotic-induced suprainfection', *Journal of Infectious Diseases*, 119, 662–665

Weller, T. M. (2000) 'Methicillin-resistant *Staphylococcus aureus* typing methods: which should be the international standard?', *Journal of Hospital Infections*, 44, 160-172

Wenzel, R. P. and Perl, T. M. (1995) 'The significance of nasal carriage of *Staphylococcus aureus* and the incidence of postoperative wound infection', *Journal of Hospital Infections*, 31, 13–24

Wertheim, H. F., Melles, D. C., Vos, M. C., van Leeuwen, W., van Belkum, A., Verbrugh, H. A. and Nouwen, J. L. (2005) 'The role of nasal carriage in *Staphylococcus aureus* infections', *Lancet Infectious Diseases*, 5, 751–762

Wesson, C. A., Deringer, J., Liou, L. E., Bayles, K. W., Bohach, G. A. and Trumble, W. R. (2000) 'Apoptosis induced by *Staphylococcus aureus* in epithelial cells utilizes a mechanism involving caspases 8 and 3', *Infection and Immunity*, 68, 2998–3001

Wesson, C. A., Liou, L. E., Todd, K. M., Bohach, G. A., Trumble, W. R. and Bayles, K. W. (1998) '*Staphylococcus aureus Agr* and *Sar* global regulators influence internalization and induction of apoptosis', *Infection and Immunity*, 66, 5238-5243

Wielders, C. L., Vriens, M. R., Brisse, S., de Graaf-Miltenburg, L. A., Troelstra, A., Fleer, A., Schmitz, F. J., Verhoef, J. and Fluit, A. C. (2001) '*In-vivo* transfer of *mecA* DNA to *Staphylococcus aureus* [corrected]', *Lancet*, 357, 1674–1675

Wild, P., McEwan, D. G. and Dikic, I. (2014) 'The LC3 interactome at a glance', *Journal of Cell Science*, 127, 1–7

Wilson, C., Lukowicz, R., Merchant, S., Valquier-Flynn, H., Caballero, J., Sandoval, J., Okuom, M., Huber, C., Brooks, T. D., Wilson, E., Clement, B., Wentworth, C. D. and Holmes, A. E. (2017) 'Quantitative and qualitative assessment methods for biofilm growth: a mini-review', *Research & Reviews. Journal of Engineering and Technology*, 6(4)

Wilson, G. S. and Miles, A. (1975) 'The mechanisms of bacterial invasion', In: *Topley and Wilson's principles of bacteriology, virology and immunity*, Baltimore, Md: The Williams & Wilkins Co., pp. 1273–1299

Wisplinghoff, H., Rosato, A. E., Enright, M. C., Noto, M., Craig, W. and Archer, G. L. (2003) 'Related clones containing *SCCmec* type IV predominate among clinically significant *Staphylococcus epidermidis* Isolates', *Antimicrobial Agents and Chemotherapy*, 47, 3574–3579

Wong-Beringer, A., Joo, J., Tse, E. and Beringer, P. (2011) 'Vancomycin-associated nephrotoxicity: a critical appraisal of risk with high-dose therapy', *International Journal of Antimicrobial Agents*, 37, 95–101

Wu, P. J., Jeyaratnam, D., Tosas, O., Cooper, B. S. and French, G. L. (2017) 'Point-of-care universal screening for meticillin-resistant *Staphylococcus aureus*: a cluster-randomized cross-over trial', *Journal of Hospital Infection*, 95, 245-252

Wu, S. W., de Lencastre, H. and Tomasz A. (2001) 'Recruitment of the *mecA* gene homologue of *Staphylococcus sciuri* into a resistance determinant and expression of the resistant phenotype in *Staphylococcus aureus', Journal of Bacteriology*, 183, 2417–2424

Wu, S., Piscitelli, C., de Lencastre, H. and Tomasz, A. (1996) 'Tracking the evolutionary origin of the methicillin resistance gene: cloning and sequencing of a homologue of *mecA* from a methicillin susceptible strain of *Staphylococcus sciuri*', *Microbial Drug Resistance*, 2, 435–441

www.mlst.net

www.sccmec.org/Pages/SCC_TypesEN.html

Yahara, K., Didelot, X., Jolley, K. A., Kobayashi, I., Maiden, M. C., Sheppard, S. K. and Falush, D. (2015) 'The landscape of realized homologous recombination in pathogenic bacteria', *Molecular Biology and Evolution*, 33, 456–471

Yamamoto, T., Takano, T., Higuchi, W., Iwao, Y., Singur, O., Reva, I., Otsuka, Y., Nakayashiki, T., Mori, H., Reva, G., Kuznetsov, V. and Potapov, V. (2012) 'Comparative genomics and drug resistance of a geographic variant of ST239 methicillin-resistant *Staphylococcus aureus* emerged in Russia', *PLoS One*, 7, e29187

Yang, F., Riedel, R., Del Pino, P., Pelaz, B., Said, A. H., Soliman, M., Pinnapireddy, S. R., Feliu, N., Parak, W. J., Bakowsky, U. and Hampp, N. (2017) 'Real-time, label-free monitoring of cell viability based on cell adhesion measurements with an atomic force microscope', *Journal of Nanobiotechnology*, 15, 23

Yang, Y., McBride, M. V., Rodvold, K. A., Tverdek, F., Trese, A. M., Hennenfent, J., Schiff, G., Lambert, B. L. and Schumock, G. T. (2010) 'Hospital policies and practices on prevention and treatment of infections caused by methicillin- resistant *Staphylococcus aureus*', *American Journal of Health-System Pharmacy*, 67, 1017–1024

Yarwood, J. M. and Schlievert, P. M. (2003) 'Quorum sensing in *Staphylococcus* infections', *The Journal of Clinical Investigation*, 112, 1620–1625

Yarwood, J. M., Bartels, D. J., Volper, E. M. and Greenberg, E. P. (2004) 'Quorum sensing in *Staphylococcus aureus* biofilms', *Journal of Bacteriology*, 186, 1838-1850

Yatim, N., Cullen, S. and Albert, M. L. (2017) 'Dying cells actively regulate adaptive immune responses', *Nature Reviews Immunology*, 17, 262–275

Yoshii, S. R. and Mizushima, N. (2017) 'Monitoring and measuring autophagy', International *Journal of Molecular Sciences*, 18, 1865

Yuan, J. and Kroemer, G. (2010) 'Alternative cell death mechanisms in development and beyond', *Genes and Development*, 24, 2592–2602

Yuan, J., Najafov, A. and Py, B. F. (2016) 'Roles of caspases in necrotic cell death', *Cell*, 167, 1693–1704

Zanger, P., Nurjadi, D., Schleucher, R., Scherbaum, H., Wolz, C., Kremsner, P. G. and Schulte, B. (2012) **'**Import and spread of Panton–Valentine leukocidin-positive *Staphylococcus aureus* through nasal carriage and skin infections in travelers returning from the tropics and subtropics', *Clinical Infectious Diseases,* 54, 483-492

Zhai, W., Wu, F., Zhang, Y., Fu, Y. and Liu, Z. (2019) 'The immune escape mechanisms of *Mycobacterium Tuberculosis', International Journal of Molecular Sciences,* 20, 340

Zhang, K., McClure, J., Elsayed, S., Louie, T. and Conly, J. M. (2005) 'Novel Multiplex PCR Assay for Characterization and Concomitant Subtyping of Staphylococcal Cassette Chromosome *mec* Types I to V in Methicillin-Resistant *Staphylococcus aureus', Journal of Clinical Microbiology,* 43, 5026–5033

Zhang, X., Li, Y., Li, B., Mao, Y., Wu, X., Zou, X., Gao, P., Yan, H., Yang, D., Ling, Y. and Chen, H. (2016) 'Three supplementary methods for analyzing cytotoxicity of *Escherichia coli* O157:H7', Journal of Microbiological Methods, 120, 34–40

Zhou, C., Bhinderwala, F., Lehman, M. K., Thomas, V. C., Chaudhari, S. S., Yamada, K. J., Foster, K. W., Powers, R., Kielian, T. and Fey, P. D. (2019) 'Urease is an essential component of the acid response network of *Staphylococcus aureus* and is required for a persistent murine kidney infection', *PLoS Pathogens*, 15, e1007538

Zhou, Y., Zhang, M., Hider, R. C. and Zhou, T. (2014) '*In vitro* antimicrobial activity of hydroxypyridinone hexadentate-based dendrimeric chelators alone and in combination with norfloxacin', *FEMS Microbiology Letters*, 355, 124–130

Ziebandt, A. K., Kusch, H., Degner, M., Jaglitz, S., Sibbald, M. J., Arends, J. P. Chlebowicz, M. A., Albrecht, D., Pantucek, R., Doskar, J., Ziebuhr, W., Broker, B. M., Hecker, M., Van Dijl, J. M., and Engelmann, S. (2010) 'Proteomics uncovers extreme heterogeneity in the *Staphylococcus aureus* exoproteome due to genomic plasticity and variant gene regulation', *Proteomics* 10, 1634–1644

Zong, Z., Peng, C. and Lu, X. (2011) 'Diversity of SCC*mec* elements in methicillin-resistant coagulase-negative staphylococci clinical isolates', *PLoS ONE*, 6(5)

Appendices

APPENDIX A: Lists of media, reagents and kits used

List of growth media

Minimum Essential Medium Eagle (EMEM)	(BioWhittaker™-Lonza BE12-611F)
Dulbecco's Modified Eagle's Medium (DMEM)	(BioWhittaker™-Lonza BE04-687F/U1)
MEM Non-Essential Amino Acid Solution (100×)	(Sigma 7145)
Fetal Bovine Serum	(Fisher Scientific 10117272)
Phosphate buffered saline (PBS)	(Sigma P4417)
CLED Agar	(Sigma 55420)
Mueller Hinton Agar	(Sigma 70191)
Accutase® solution	(Sigma A6964)

Antibiotics and antimicrobial agents

Vancomycin	(Sigma V2002)
Ciprofloxacin	(Sigma 17850)
Gentamicin	(Sigma G1914)
Tetracycline	(Sigma 87128)
Cefoxitin	(Sigma C4786)
Fusidic acid	(Sigma Y0001411)

Antibiotic discs

Cefoxitin 30 µg	(Fisher Scientific CT0119B)

Linezolid 10 µg	(Fisher Scientific CT1649B)
Fusidic acid 10 µg	(Fisher Scientific CT0023B)
Tetracycline 30 µg	(Fisher Scientific CT0054B)
Amikacin 30 µg	(Fisher Scientific CT0107B)
Ciprofloxacin 5 µg	(Fisher Scientific CT0425B)
Erythromycin 15 µg	(Fisher Scientific CT0020B)
Clindamycin 2 µg	(Fisher Scientific CT0064B)
Tigecycline 15 µg	(Fisher Scientific CT1841B)
Quinupristin/Dalfopristin 15 µg	(Fisher Scientific CT1644B)
Rifampicin 5 µg	(Fisher Scientific CT0207B)
Gentamicin 10 µg	(Fisher Scientific CT0024B)
Kits	
QIAamp DNA Micro Kit	(QIAGEN 56304)
QIAquick Gel Extraction Kit	(QIAGEN 28704)
Cytotoxicity Detection Kit (LDH)	(ROCHE 11644793001)
Reagents for flow cytometry	
Annexin V FITC	(BioLegend 640906)
Annexin V Binding Buffer	(BioLegend 422201)
Cell staining buffer	(BioLegend 420201)
Zombie NIR	(BioLegend 423106)

Reagents for PCR and gel electrophoresis

Agarose	(Fisher Scientific 10776644)
GeneRuler 1 kb DNA Ladder	(Thermo Scientific™ SM0311)
DNA loading dye	(Thermo Scientific™ R0631)
dNTP Mix	(Thermo Scientific™ R0241)
Taq DNA Polymerase	(Thermo Scientific™ EP0401)
SafeView Nucleic Acid Stain	(NBS-Biologicals NBS-SV1)

General reagents

Ethylenediaminetetraacetic acid (EDTA) (10×)	(Sigma E9884)
Acetone	(Fisher Scientific 10045800)
Hematoxylin	(Sigma H9627)
CelLytic™ M	(Sigma C2978)
Virkon	(Fisher Scientific 12358667)
Ethanol	(Fisher Scientific BP2818)
Dimethyl Sulfoxide (DMSO)	(Sigma D2650)

APPENDIX B: Recipes

Media recipes

1X PBS: Every 100ml contained 0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.137 M sodium chloride.

EMEM for KB epithelial cell line (v/v): EMEM 89 %, NEAA 1 %, and FBS 10 %.

DMEM for A549 cell line (v/v): DMEM 89 %, NEAA 1 %, and FBS 10 %.

Luria Bertani broth (w/v): 10g tryptone, 5g yeast extract and 10g NaCl in 1000 ml distilled water. pH adjusted to 7.5 before autoclaving at 121ºC for 15 min.

CLED agar (w/v): 36.15 g of CLED agar dissolved in 1000 ml distilled water prior to autoclaving at 121ºC for 15 min.

Solvents used in preparing stock solutions of antimicrobial agents

Antimicrobial agents (Solute)	Solvent
Vancomycin	Water
Ciprofloxacin	0.05mM HCl
Gentamicin	Water
Cefoxitin	Water
Tetracycline	Water
Fusidic acid	1% Ethanol
Iron chelator 75	50% DMSO
Iron chelator 70	50% DMSO
Iron chelator 71	20% DMSO
Iron chelator 85	20% DMSO
Iron chelator 81	Water

Buffers for agarose gel electrophoresis

50X TAE buffer: 242g of tris base was dissolved in 750ml deionised water. 100ml of 0.5M EDTA and 57.1 ml of acetic acid were added to the dissolved tris base and the final volume made up to 1000 ml with deionised water.

1X TAE buffer: 20 ml of 50X TAE buffer was made up to 1000 ml with distilled water.

1% Agarose gel: 50 mg agarose was dissolved in 50 ml 1X TAE buffer at 50 ºC following which 1.5 µl of SafeView was added and mixed. This molten mix was then poured into the gel casting tray of the electrophoresis apparatus and left to set/solidify.

Primer preparation

Each tube of primer was spun at high speed for at least 120 secs to ensure that no lyophilised primer pellet was stuck to the cap. Afterward, each primer was resuspended in deionised water to make a 100 µM primer stock. The number of nmoles of primer in each tube was multiplied by 10 to determine the volume of deionised water to add to make a 100 µM stock. For instance, 250 µl of water was added to 25.0 nmol of lyophilised primer to get a 100 µM stock. Primer stocks were subsequently diluted to 10 µM working concentrations and all primers were stored at -20ºC.

Disinfections used in the laboratory

1 % virkon (w/v): 1 g in 100 ml water

70 % ethanol (v/v): 70 ml in 100 ml H_2O

APPENDIX C: Cell lines and bacterial isolates

List of Cell lines

KB skin epithelial cell line (carcinoma cells)

A549 lung epithelial cell line (carcinoma cells)

Bacterial isolates

Six clinical MRSA isolates including MRSA NCTC 12493 and *S. aureus* NCTC 6571 reference strains were obtained from Colchester General Hospital.

All 16 clinical Nigerian MRSA isolates were obtained from

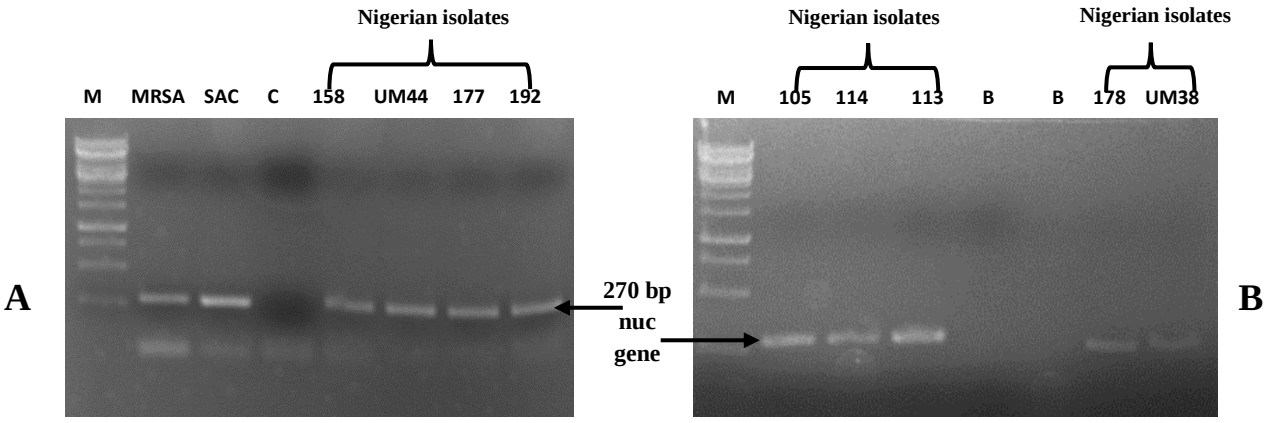
Bacterial glycerol stocks for long term storage

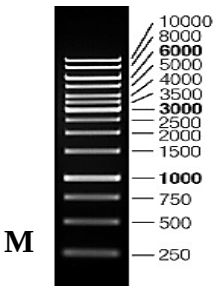
Using a disposal sterile loop, about 20-30 isolated colonies of bacteria were picked from a CLED agar plate and inoculated in 10 ml of bacterial freezing medium i.e. 70 % LB broth and 30 % glycerol (v/v). Aliquots of the mixture of freezing medium with bacteria were then stored in cryovials at -80 °C.

Re-culturing bacteria

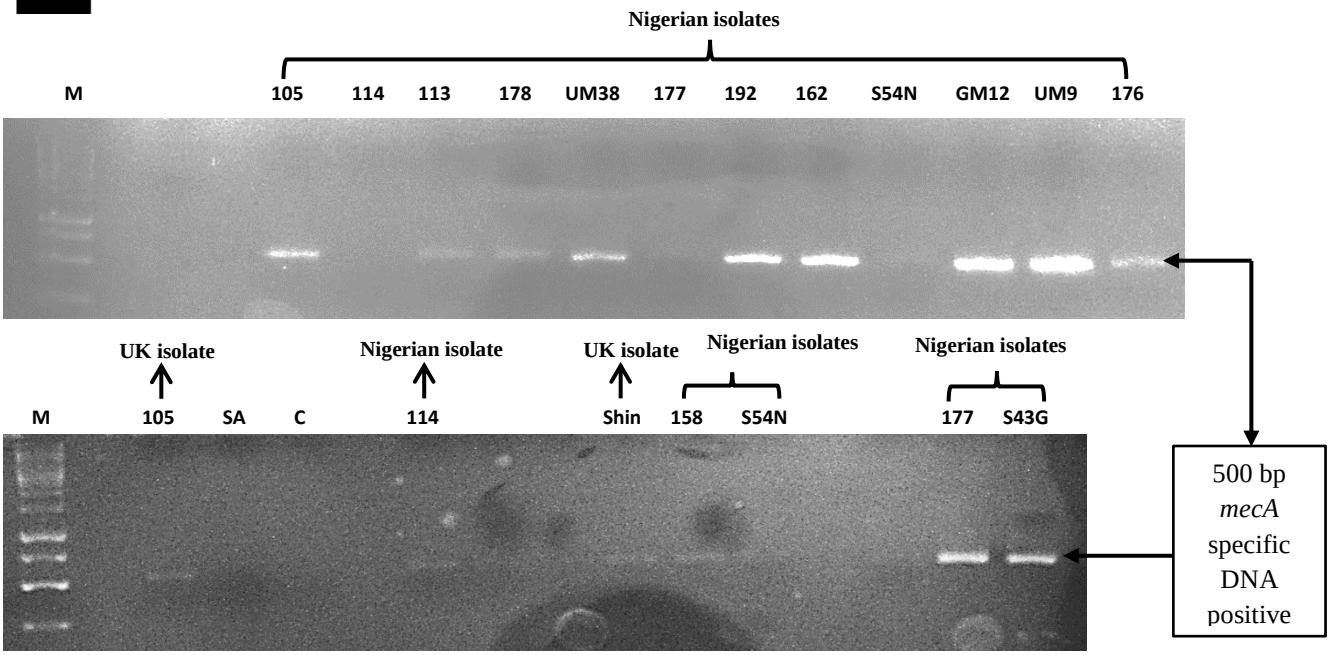
To obtain some of the frozen bacteria, a sterile disposable loop was used to scrap the surface of the frozen mixture of freezing medium with bacteria in a cryovial. This was then streaked onto a fresh agar plate which was subsequently incubated overnight at 37°C. Following this, morphological characteristics of the colonies on the plates were examined to ensure that the culture was pure and then biochemical tests were also carried out to further confirm identities of the isolates.

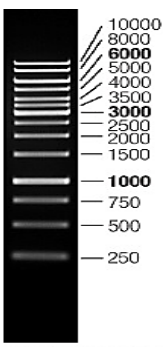
APPENDIX D: Supplementary figures



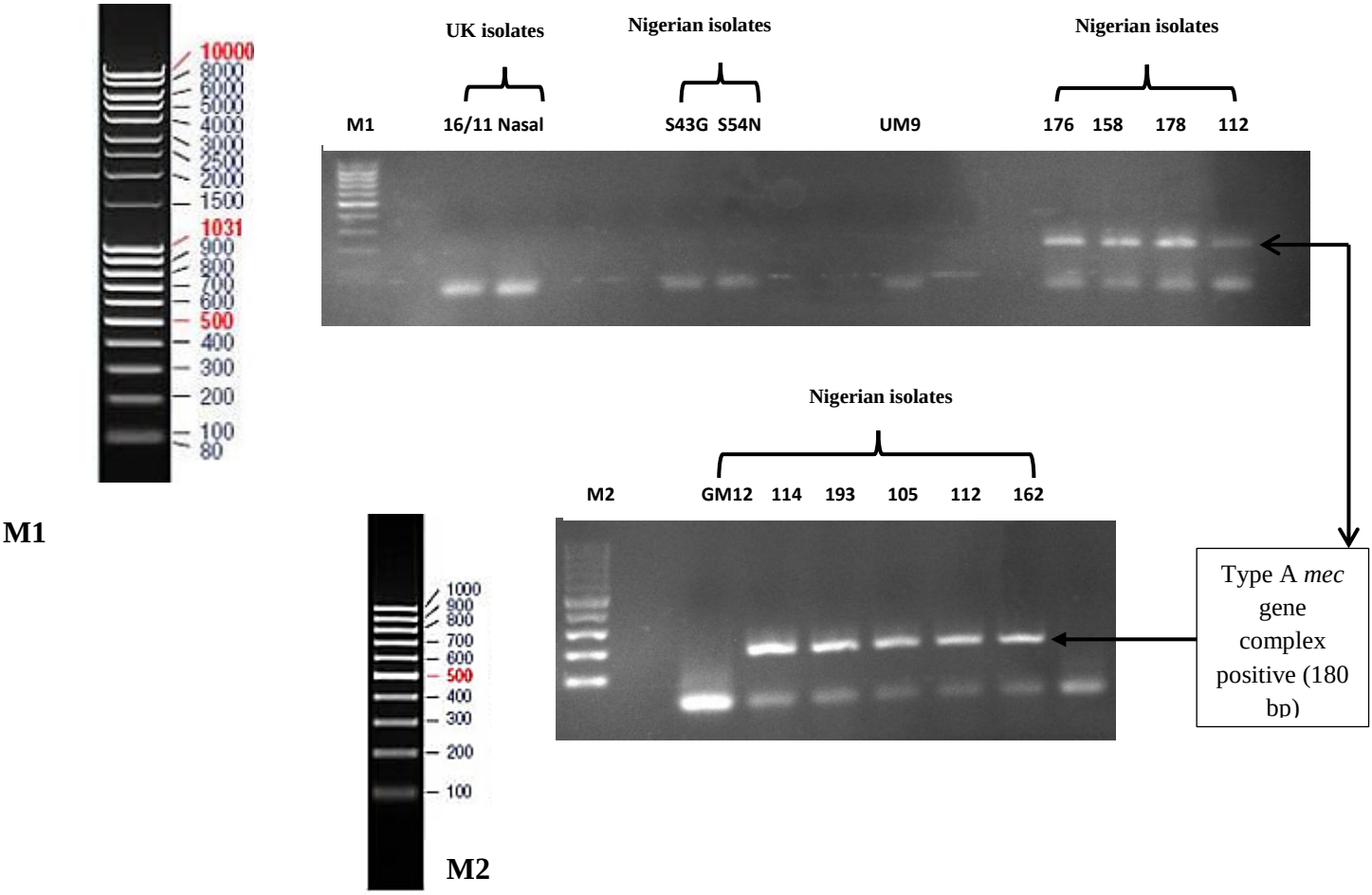


D.1: Gels showing products obtained from the PCR to amplify the ~ 270 bp region of the nuc gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **B:** Empty wells, **M**: 1 kb DNA ladder (Thermo Scientific).

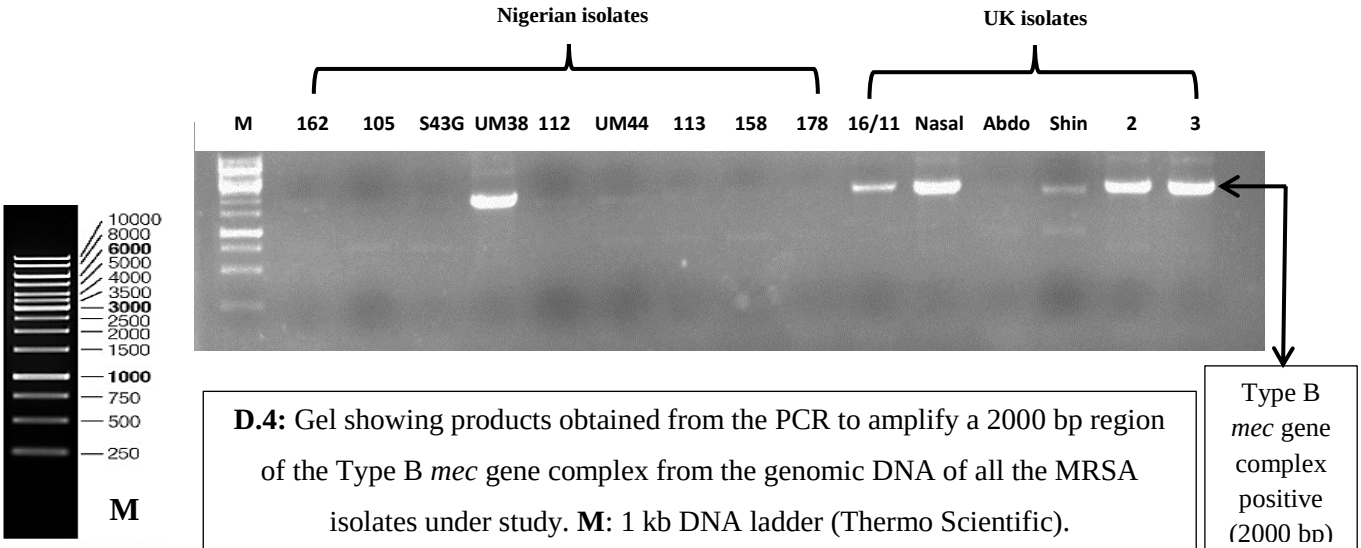




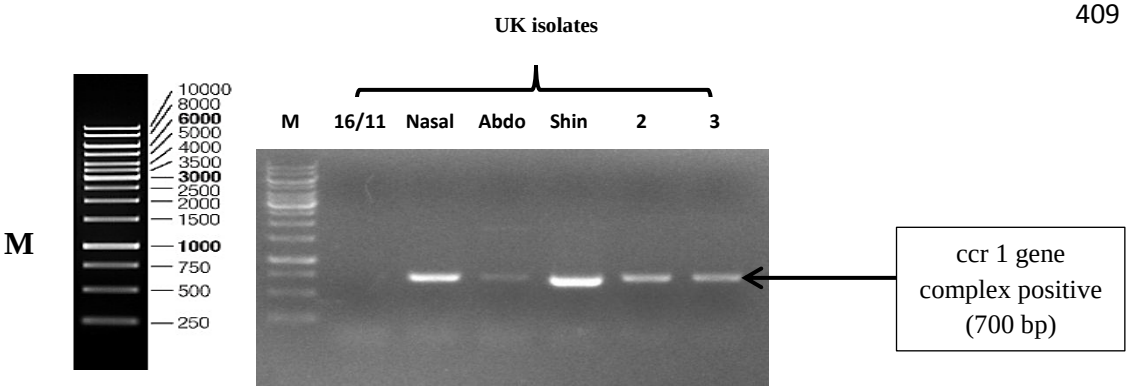
D.2: Gels showing products obtained from the PCR to amplify a 500 bp region of the *mecA* gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 1 kb DNA ladder (Thermo Scientific).



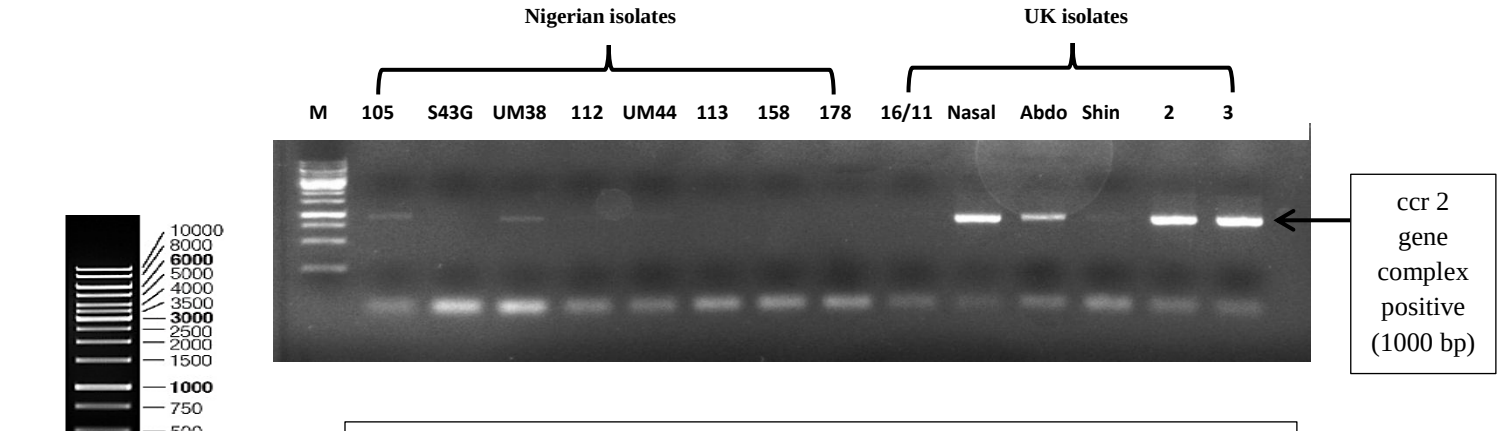
D.3: Gels showing products obtained from the PCR to amplify a 180 bp region of the Type A *mec* gene complex from the genomic DNA of all MRSA isolates under study. **M1**: MassRuler DNA Ladder (Thermo Scientific): **M2:** 100 bp DNA ladder (Thermo Scientific).



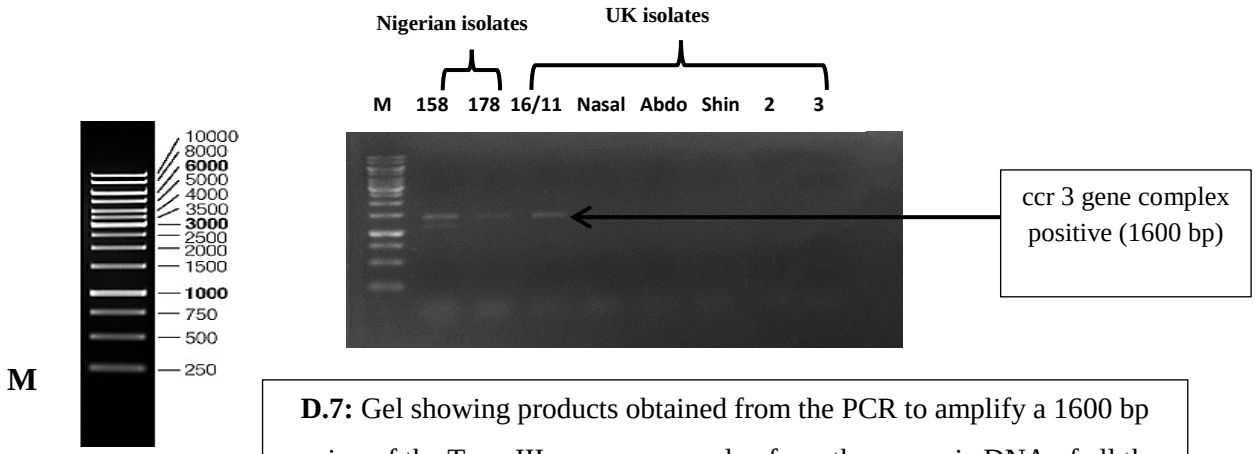
D.4: Gel showing products obtained from the PCR to amplify a 2000 bp region of the Type B *mec* gene complex from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



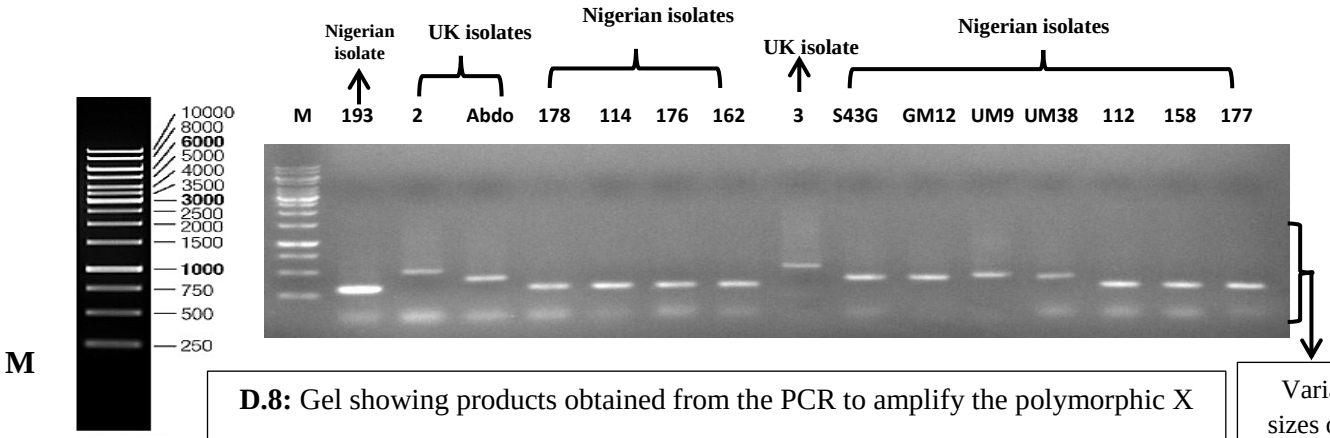
D.5: Gel showing products obtained from the PCR to amplify a 700 bp region of the Type I *ccr* gene complex from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



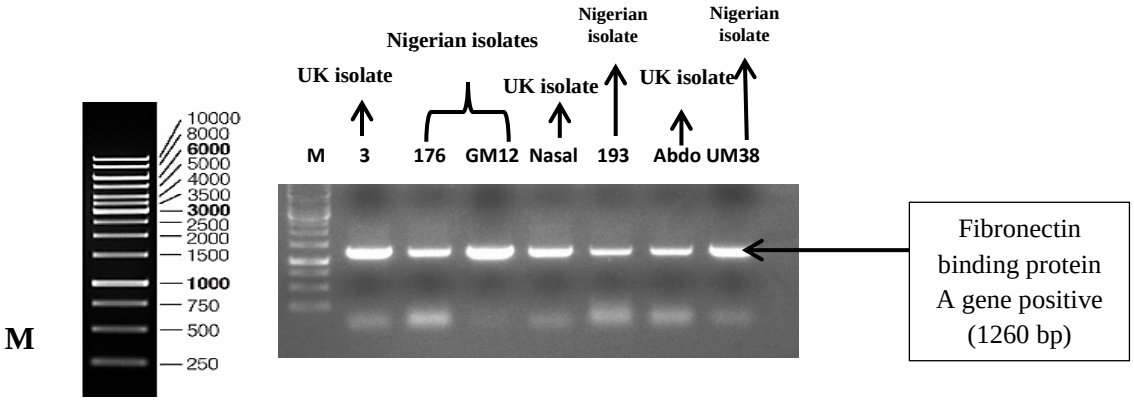
D.6: Gel showing products obtained from the PCR to amplify a 1000 bp region of the Type II *ccr* gene complex from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



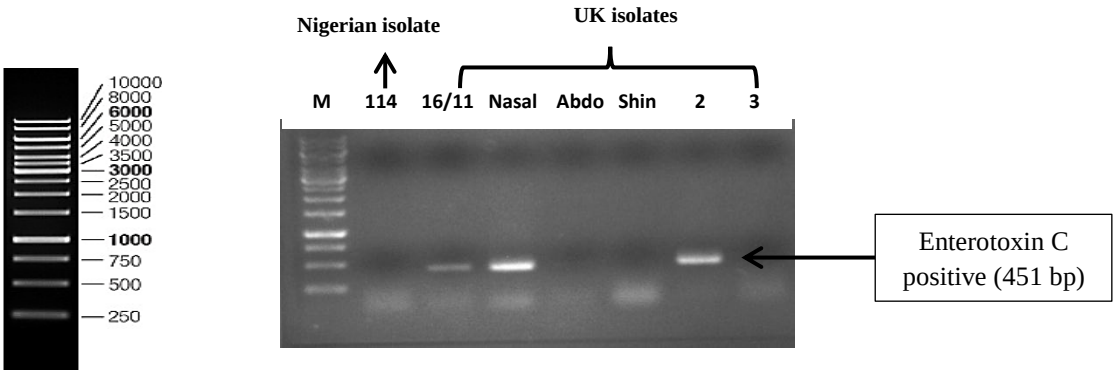
D.7: Gel showing products obtained from the PCR to amplify a 1600 bp region of the Type III *ccr* gene complex from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



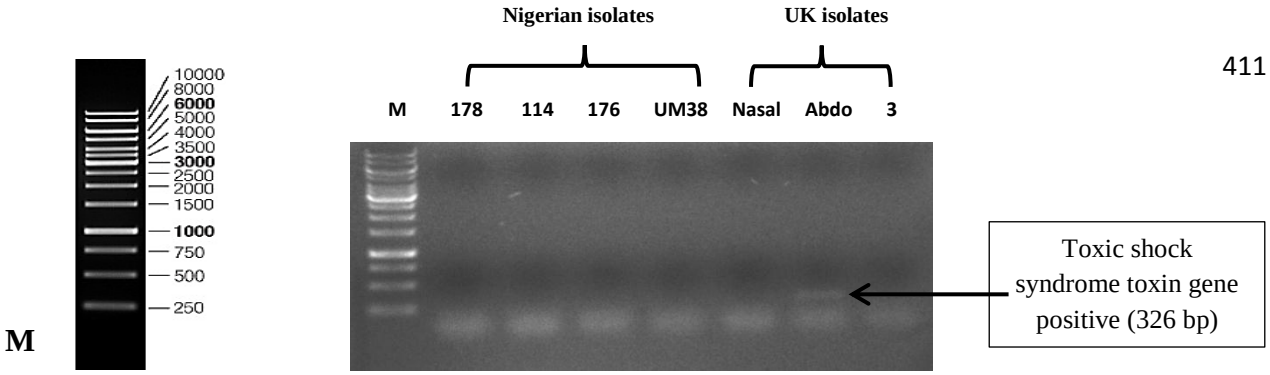
D.8: Gel showing products obtained from the PCR to amplify the polymorphic X region of the spa gene from the genomic DNA of all MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



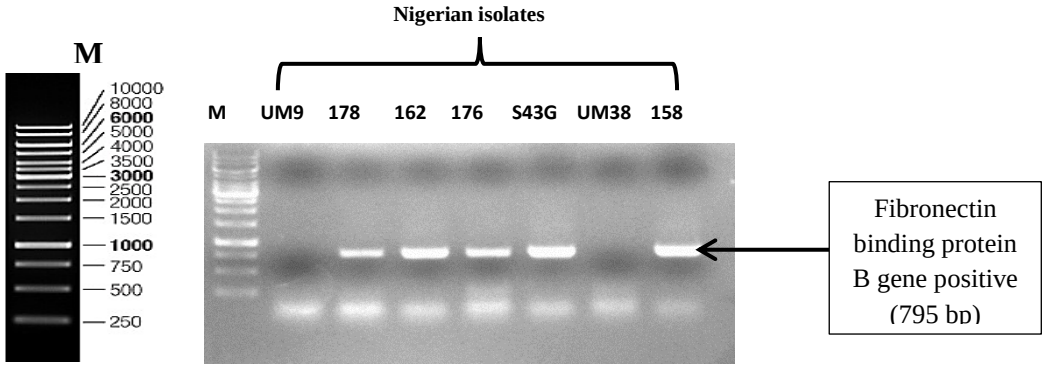
D.9: Gel showing products obtained from the PCR to amplify a 1260 bp region of the fibronectin binding protein A gene from the genomic DNA of all MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



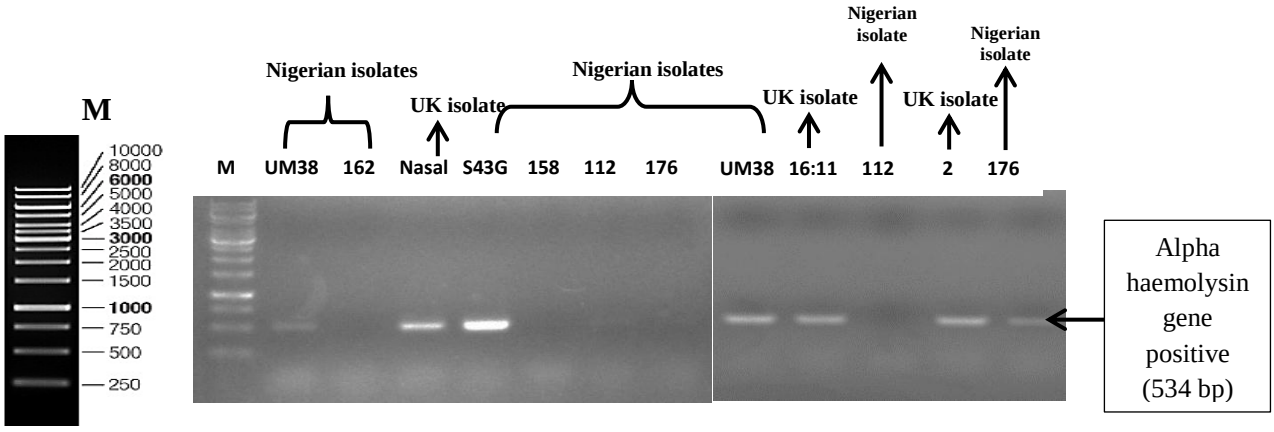
D.10: Gel showing products obtained from the PCR to amplify a 451 bp region of the Enterotoxin C gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



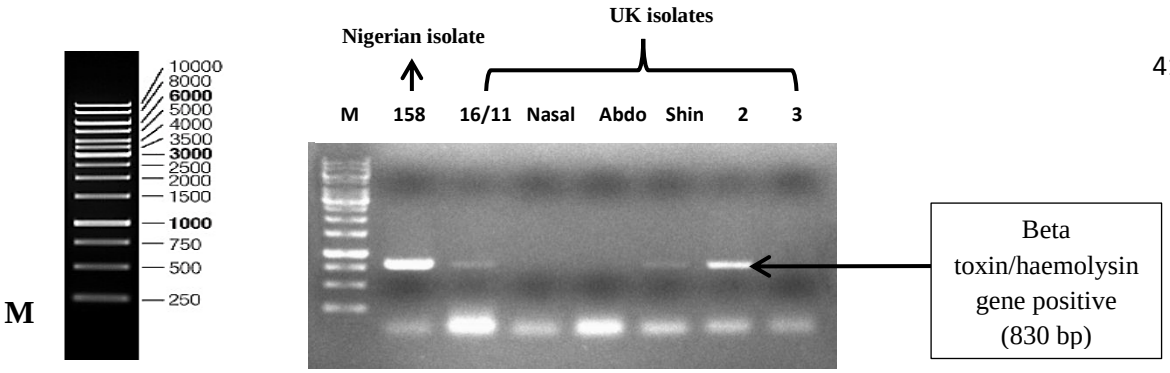
D.11: Gel showing products obtained from the PCR to amplify a 326 bp region of toxic shock syndrome toxin gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



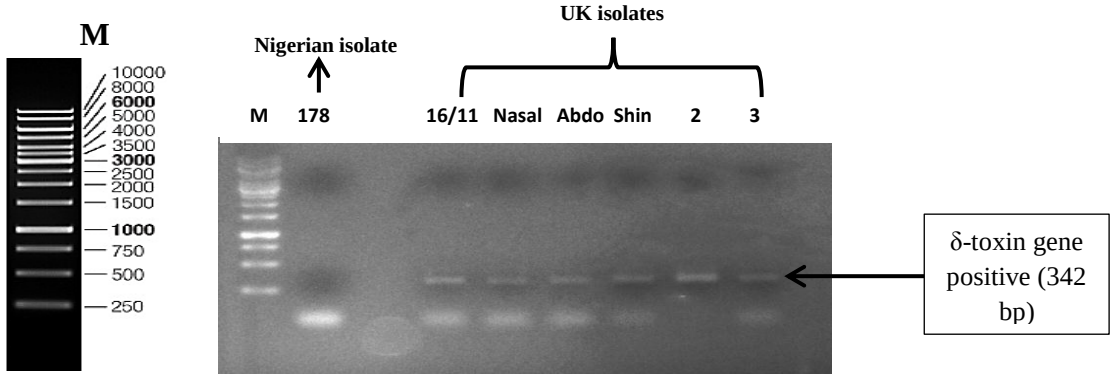
D.12: Gel showing products obtained from the PCR to amplify a 795 bp region of the fibronectin binding protein B gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



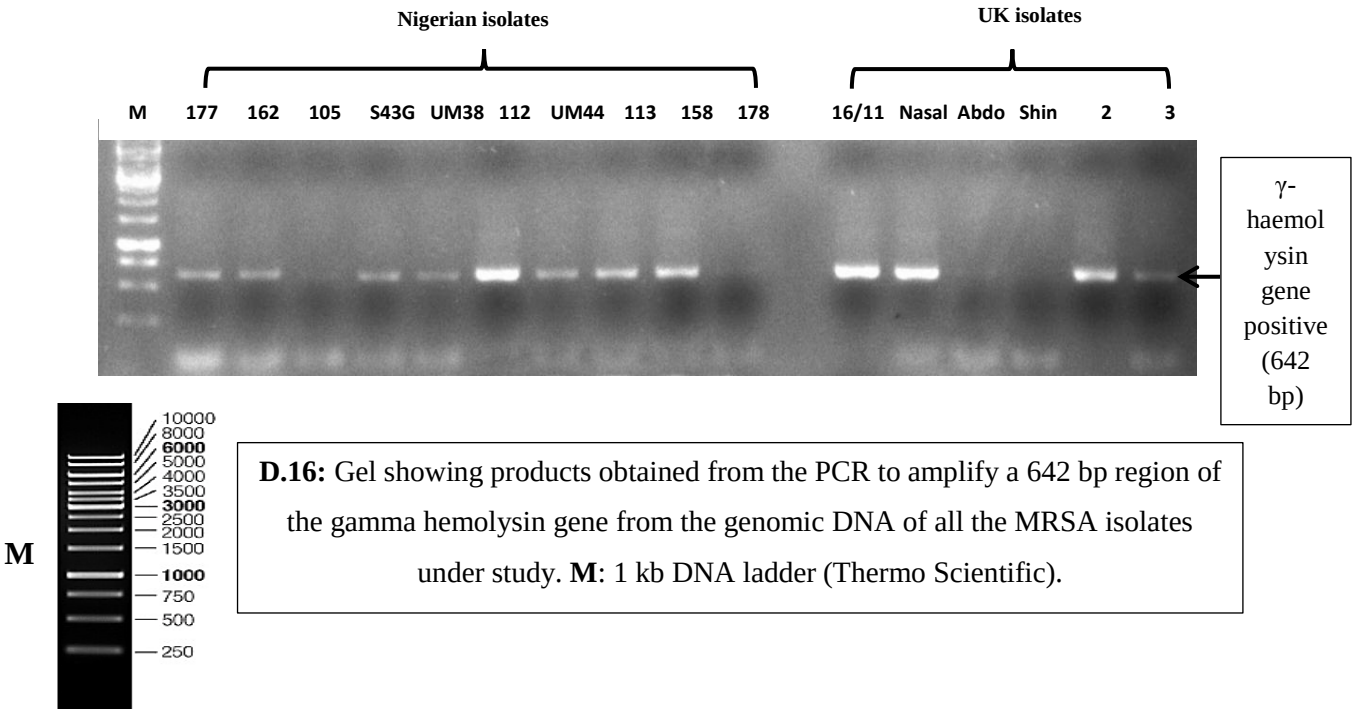
D.13: Gels showing products obtained from the PCR to amplify a 534 bp region of alpha toxin gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



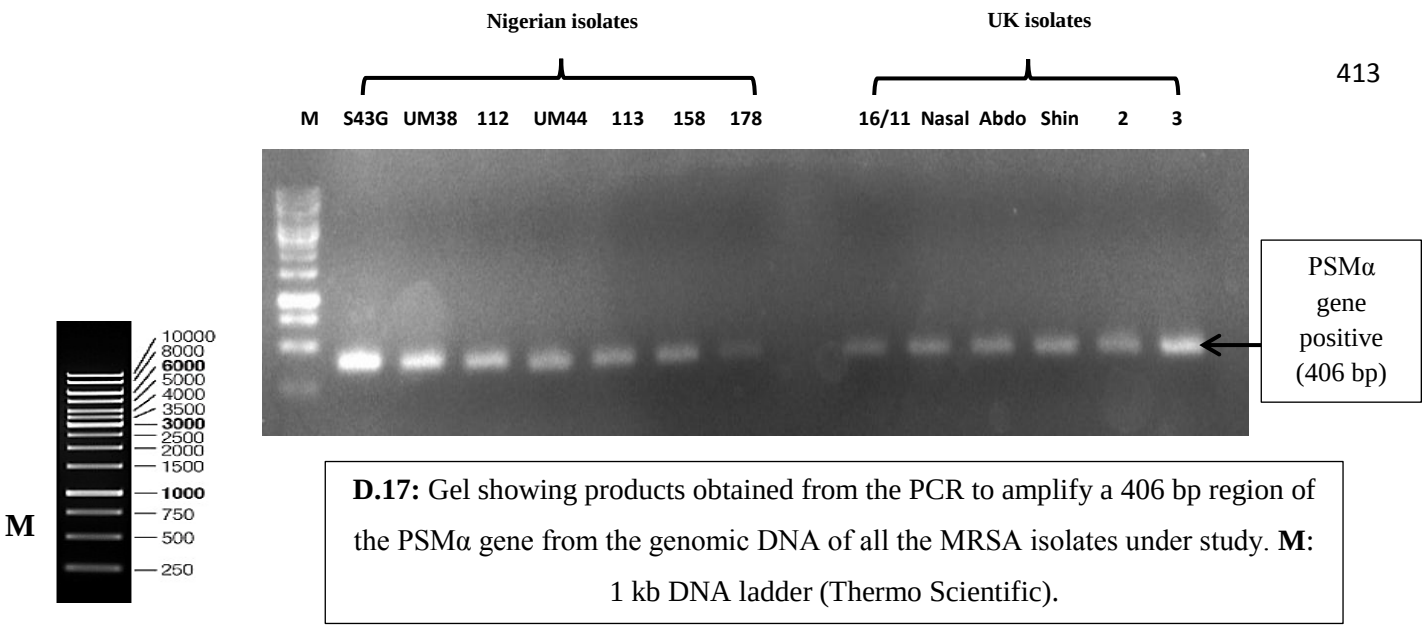
D.14: Gel showing products obtained from the PCR to amplify an 830 bp region of the beta toxin gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



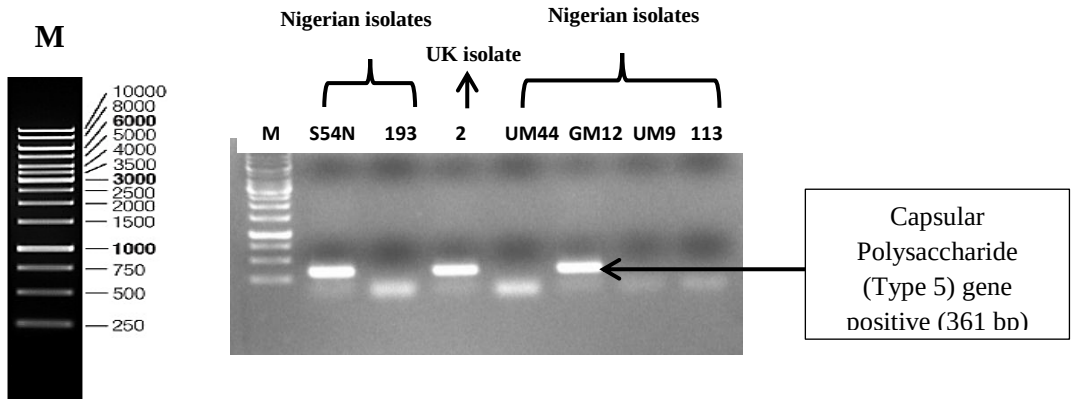
D.15: Gel showing products obtained from the PCR to amplify a 342 bp region of the delta haemolysin gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



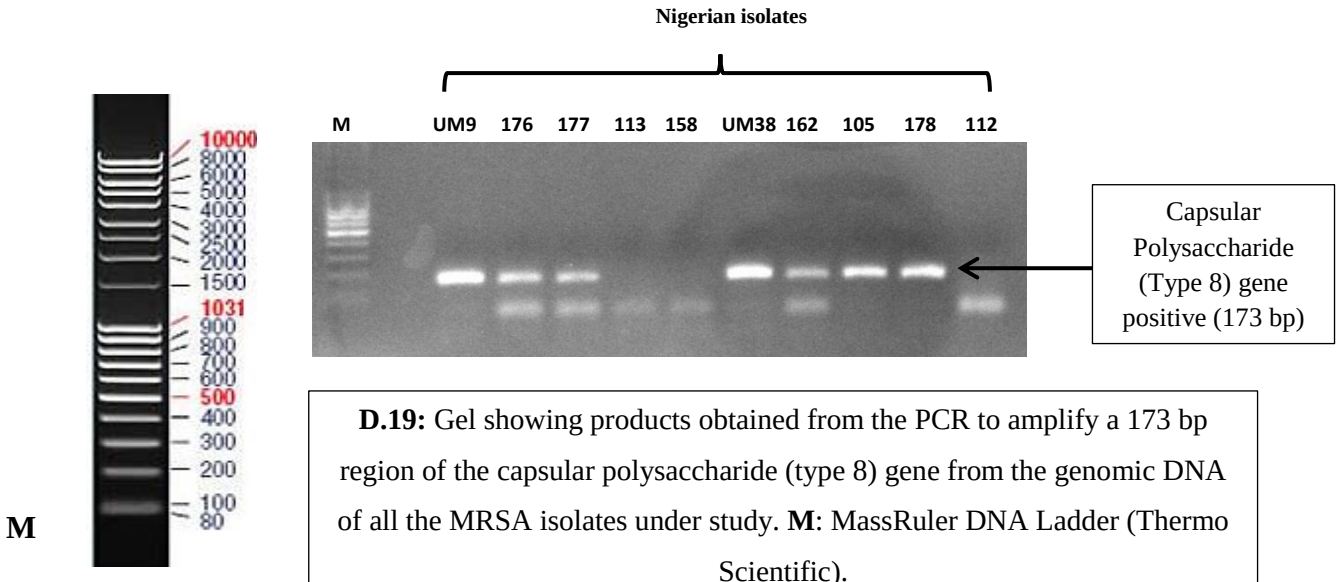
D.16: Gel showing products obtained from the PCR to amplify a 642 bp region of the gamma hemolysin gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



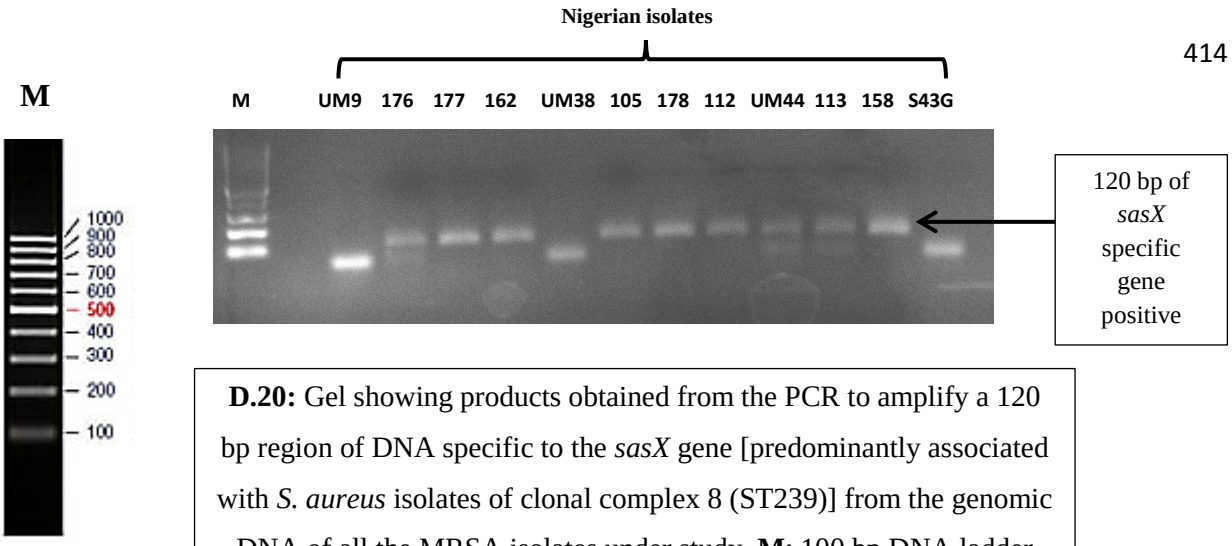
D.17: Gel showing products obtained from the PCR to amplify a 406 bp region of the PSMα gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



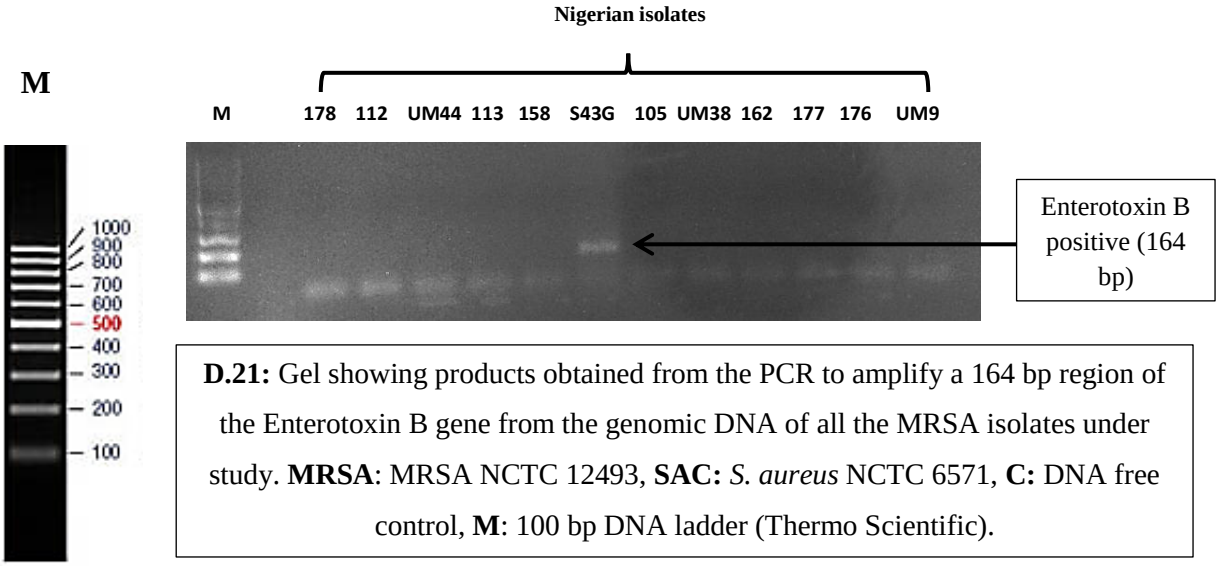
D.18: Gel showing products obtained from the PCR to amplify a 361 bp region of the capsular polysaccharide (type 5) gene from the genomic DNA of all the MRSA isolates under study. **M**: 1 kb DNA ladder (Thermo Scientific).



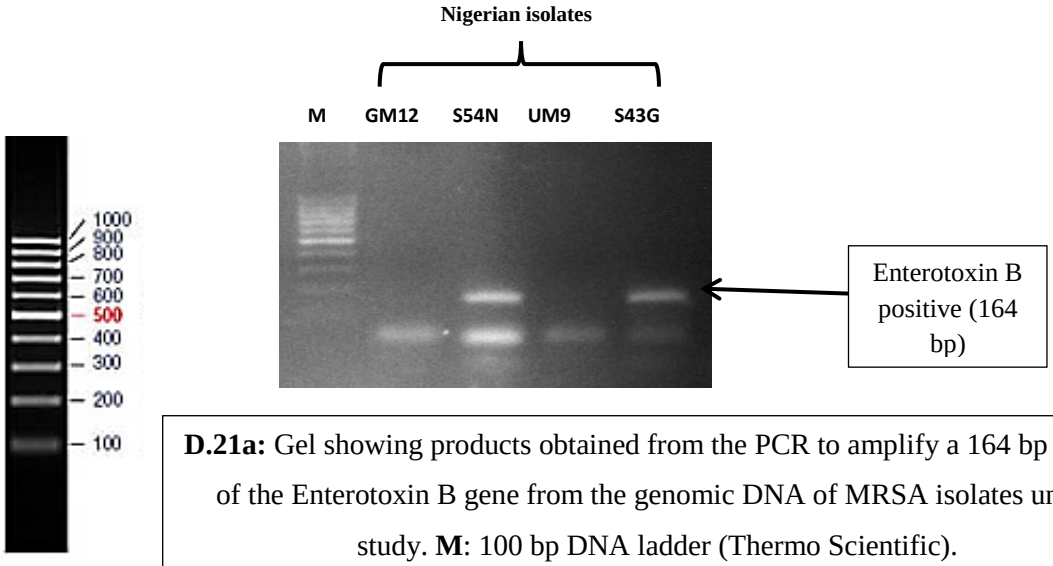
D.19: Gel showing products obtained from the PCR to amplify a 173 bp region of the capsular polysaccharide (type 8) gene from the genomic DNA of all the MRSA isolates under study. **M**: MassRuler DNA Ladder (Thermo Scientific).



D.20: Gel showing products obtained from the PCR to amplify a 120 bp region of DNA specific to the *sasX* gene [predominantly associated with *S. aureus* isolates of clonal complex 8 (ST239)] from the genomic DNA of all the MRSA isolates under study. **M**: 100 bp DNA ladder (Thermo Scientific).



D.21: Gel showing products obtained from the PCR to amplify a 164 bp region of the Enterotoxin B gene from the genomic DNA of all the MRSA isolates under study. **MRSA**: MRSA NCTC 12493, **SAC:** *S. aureus* NCTC 6571, **C:** DNA free control, **M**: 100 bp DNA ladder (Thermo Scientific).



D.21a: Gel showing products obtained from the PCR to amplify a 164 bp region of the Enterotoxin B gene from the genomic DNA of MRSA isolates under study. **M**: 100 bp DNA ladder (Thermo Scientific).

APPENDIX E: Supplementary tables

E.1: Primers used in this study

Primer	Sequence (5'-3')	DNA fragments amplified	Reference
Primers to confirm identities of the MRSA isolates			
nuc 1 nuc 2	GCGATTGATGGTGATACGGTT AGCCAAGCCTTGAACGAACTAAAGC	~270 bp of *S. aureus* thermonuclease-nuc gene (nuc 1)	Ali *et al*., 2014.
mec1 mec 2	TCCAGATTACAACTTCACCAGG CAATTCATATCTTGTAACG	500 bp *mecA* specific DNA	Khan *et al*., 2012
Primers for Clonal typing			
1 CC30 2 CC30	AGGGTTTGAAGGCGAATGGG CAACAGAATAATTTTTTAGTTC	203 bp specific to MRSA isolates of the CC30 lineage	Cockfield *et al*., 2007
1 CC22 2 CC22	AGGGTTTGAAGGCGAATGGG TCAGAGCTCAACAATGATGC	990 bp specific to MRSA isolats of CC22 lineage	
sas 1 sas 2	ATTGAAGCTCAGACTCCTAG GTTATCAGTTGTAGCAGTAGT	120 bp specific to the *sasX* gene	Monecke *et al*., 2014

E.1: Primers used in this study (contd.)

Primer	Sequence (5'-3')	DNA fragments amplified	Reference
Primers for SCC*mec* typing			
mec A1 mec A2	CAAGTGATTTGAAACCGCCT CAAAAGGACTGGACTGGAGTCCAAA	180 bp of the type A *mec* gene complex	Okuma *et al*., 2002
mec B1 mec B2	ACCGCCACTCATAACATAAGGAA TATACCAACCCGACAAC	2000 bp of the type B *mec* gene complex	
Type I-F Type I-R	GCTTTAAAGAGTGTCGTTACAGG GTTCTCTCATAGTATGACGTCC	613 bp of SCC*mec* I	McClure-Warnier *et al*., 2013
Type II-F Type II-R	CGTTGAAGATGATGAAGCG CGAAATCAATGGTTAATGGACC	398 bp of SCC*mec* II	
Type II-F2 Type II-R2	TAGCTTATGGTGCTTATGCG GTGCATGATTTCATTTGTGGC	128 bp of SCC*mec* II, VIII	
Type III-F Type III-R	CCATATTGTGTACGATGCG CCTTAGTTGTCGTAACAGATCG	280 bp of the Mercury element of SCC*mec* III	
Type III-F5 Type III-R6	TTCTCATTGATGCTGAAGCC GTGTAATTTCTTTTGAAAGATATGG	257 bp of SCC*mec* III, IIIA	
Type IVa-F Type IVa-R	GCCTTATTCGAAGAAACCG CTACTCTTCTGAAAAGCGTCG	776 bp of SCC*mec* Iva	
Type IVb-F Type IVb-R	TCTGGAATTACTTCAGCTGC AAACAATATTGCTCTCCCTC	493 bp of SCC*mec* IVb, IIA, IIB IIC, IIE	
Type IVc-F2 Type IVc-R2	CCTGAATCTAAAGAGATACACCG GGTTATTTTCATAGTGAATCGC	200 bp of SCC*mec* IVc	
Type IVd-F5 Type IVd-R6	CTCAAAATACGGACCCCAATACA TGCTCCAGTAATTGCTAAAG	881 bp of SCC*mec* IVd	
Type IVE-F3 Type IVE-R4	CAGATTCATCATTTCAAAGGC AACAACTATTAGATAATTTCCG	175 bp of SCC*mec* IVE, IVF	
Type V-F Type V-R	GAACATTGTTACTTAAATGAGCG TGAAAGTTGTACCCTTGACACC	325 bp of SCC*mec* V	
ccr4-Fd ccr4-R5 ccr4-R6	ATCGCTCATTATGGATACYGC CCATTTTTTGATAACCTGAACG CTATTTTTTTATAGCCTGAACG	106 bp of SCC*mec* IIA, IIB, IIC, IIE, IVE, IVF, VI, VIII	
ccr A ccr 1b	ATTGCCTTGATAATAGCCTCT AACCTATATCATCAATCAGTACGT	700 bp of the type 1 ccr gene complex	Zhang *et al*., 2005
ccr A ccr 2b	ATTGCCTTGATAATAGCCTCT TAAAGGCATCAATGCACAAACACT	1000 bp of the type 2 ccr gene complex	
ccr A ccr 3b	ATTGCCTTGATAATAGCCTCT AGCTCAAAAGCAAGCAATAGAAT	1600 bp of the type 3 ccr gene complex	

E.1: Primers used in this study (contd.)

Primer	Sequence (5'-3')	DNA fragments amplified	Reference
Primers specific to various virulence factors			
Luk-PV-1 Luk-PV-2	ATCATTAGGTAAAATGTCTGGACATGATCCA GCATCAAGTGTATTGGATAGCAAAAGC	433 bp of the Panton Valentine Leukocidin (PVL) gene	McClure *et al*., 2006
Spa1 Spa2	TAAAGACGATCCTTCGGTGAGC CAGCAGTAGTGCCGTTTGCTT	Polymorphic X region of the spa gene (Variable sizes)	Strommenger *et al*., 2008
entA1 entA2	GGTTATCAATGTGCGGGTGG CGCCACTTTTTTCTCTTCGG	102 bp of the enterotoxin A gene	Chapaval *et al*., 2006
entB1 entB2	GTATGGTGGTGTAACTGAGC CCAAATAGTGACGAGTTAGG	164 bp of the enterotoxin B gene	
entC1 entC2	AGATGAAGTAGTTGATGTGTATGG CACACTTTTAGAATCAACCG	451 bp of the enterotoxin C gene	
entD1 entD2	CCAATAATAGGAGAAAATAAAAG ATTGGTATTTTTTTTCGTTC	278 bp of the enterotoxin D gene	
entE1 entE2	AGGTTTTTTCACAGGTCATCC CTTTTTTTTCTTCGGTCAATC	209 bp of the enterotoxin E gene	
tst1 tst2	ACCCCTGTTCCCTTATCATC TTTTCAGTATTTGTAACGCC	326 bp of the toxic shock syndrome toxin (TSST-1) gene	
Cap5A Cap5B	GTCAAAGATTATGTGATGCTACTGAG ACTTCGAATATAAACTTGAATCAATGTTATACAG	361 bp of capsular polysaccharide serotype 5	Verdier *et al*., 2007
Cap8A Cap8B	GCCTTATGTTAGGTGATAAACC GGAAAAACACTATCATAGCAGG	173 bp of capsular polysaccharide serotype 8	
hla1 hla2	GGTTTAGCCTGGCCTTC CATCACGAACTCGTTCG	534 bp of the alpha toxin gene	Booth *et al*., 2001
hlb1 hlb2	GCC AAA GCC GAA TCT AAG CGC ATA TAC ATC CCA TGG	Approximately 830 bp of the beta toxin gene	
fnbA1 fnbA2	GCGGAGATCAAAGACAA CCATCTATAGCTGTGTGG	Approximately 1260 bp of the fibronectin binding protein A gene	
fnbB1 fnbB2	GGAGAAGGAATTAAGGCG GCCGTCGCCTTGAGCGT	Approximately 795 bp of the fibronectin binding protein B gene	
hld 1 hld 2	TGTTCACTGTGTCGATAATCC CTCTCCTYACTGTYATTATACG	342 bp of the δ-Haemolysin gene	Li *et al*., 2009
hlg 1 hlg 2	ATGGATGTCACTCATGCC GTATTTCCATTAAGTCCACCAG	642 bp of the γ-Haemolysin gene	
ssp 1 ssp 2	GGAGGTTTTTAGATGAAAGG CGCCATTGTCTGGATTATCAGG	988 bp of the serine protease gene	
PSMα 1 PSMα 2	ATGGGTATCATCGCTGGCATCATTAAAGTTA TTTTGCGAAAATGTCGATAATTGCTTTGAT	406 bp of the phenol soluble modulin α gene	

E.2: PCR cycling parameters

DNA amplified	**Cycling Parameters**
nuc gene	Initial denaturation step at 95ºC for 5 mins followed by 37 cycles of denaturation at 95ºC for 30 secs, annealing at 55ºC for 30 secs and extension at 72ºC for 60 secs. This was followed by a final extension step at 72ºC for 10 mins.
mecA specific DNA	Initial denaturation step at 94˚C for 4 mins followed by 35 cycles of denaturation at 94˚C for 30 secs, annealing at 53˚C for 30 secs and extension at 72˚C for 1 minute. This was followed by a final extension step at 72˚C for 4 minutes.
CC30 specific DNA	Initial denaturation at 94˚C for 4 minutes followed by 30 cycles of denaturation at 94˚C for 30 seconds, annealing at 50˚C for 30 seconds and extension at 72˚C for 1 minute. Final extension at 72˚C for 4 minutes
CC22 specific DNA	
sasX gene	
Type 1 *ccr* gene complex	
Type 2 *ccr* gene complex	
Type 3 *ccr* gene complex	
Class A *mec* gene complex	
Class B *mec* gene complex	
SCC*mec* I	Initial denaturation at 94˚C for 4 minutes followed by 35 cycles of denaturation at 94˚C for 30 seconds, annealing at 53˚C for 30 seconds and extension at 72˚C for 1 minute. Final extension at 72˚C for 4 minutes.
SCC*mec* II	
SCC*mec* II, VII	
Mercury element of SCC*mec* III	
SCC*mec* III, IIIA	
SCC*mec* IVa	
SCC*mec* IVb, IIA, IIB, IIC, IIE	
SCC*mec* IVc, IVe	
SCC*mec* IVd	
SCC*mec* IVE, IVF	
SCC*mec* V	
SCC*mec* IIA, IIB, IIC, IIE, IVE, IVF, VI, VIII	
PVL	
Polymorphic X region of the spa gene	
Phenol soluble modulin α	
Serine protease	
Haemolysins	

E.2: PCR cycling parameters (contd.)

DNA amplified	Cycling Parameters
Enterotoxin A	Initial denaturation at 96˚C for 5 minutes followed by 35 cycles of denaturation at 94˚C for 2 min, annealing at 54˚C for 2 minutes and extension at 72˚C for 1 minute. Final extension at 72° C for 7 minutes
Enterotoxin B	
Enterotoxin C	
Enterotoxin D	
Enterotoxin E	
Toxic shock syndrome toxin	
Capsular polysaccharide serotype 5	Initial denaturation at 94˚C for 5 minutes followed by 25 cycles of denaturation at 94˚C for 30 seconds, annealing at 55˚C for 30 seconds, and extension at 72˚C for 1 minute. Final extension at 72˚C for 5 minutes.
Capsular polysaccharide serotype 8	
Fibronectin-binding protein A	Initial denaturation at 95°C for 3 minutes followed by 40 cycles of denaturation at 95°C for 15 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 80 seconds (fnbA)/60 second (fnbB). Final extension at 72°C for 5 minutes.
Fibronectin-binding protein B	

E.3: Distribution of the *fnbA* and *fnbB* genes in the UK and Nigerian isolates used in the interaction assays

***fnbA*:** fibronectin binding protein A
***fnbB*:** fibronectin binding protein B

Isolate	Genes	
	fnbA	***fnbB***
UK isolates		
16/11	+	+
Nasal	+	
Abdo	+	
Shin	+	
2	+	
3	+	
Nigerian isolates		
GM12	+	+
S54N	+	+
114	+	+
193	+	+
UM9	+	
176	+	+
177	+	+
162	+	+

E.4: Distribution of the haemolysin (α, β, γ) and the δ-toxin genes in the UK and Nigerian isolates used in the cytotoxicity and flow cytometer experiments

***hla*:** alpha toxin/haemolysin
hlb: beta toxin/haemolysin
δ*-toxin*: delta toxin
hlγ; gamma toxin/haemolysin

Isolates	Genes			
	hla	***hlb***	***δ*-toxin**	***hlγ***
UK isolates				
16/11	+	+	+	+
Nasal	+		+	+
Abdo	+		+	
Shin	+	+	+	
2	+	+	+	+
3	+		+	+
Nigerian isolates				
GM12	+	+	+	+
S54N	+	+	+	+
114		+		+
193		+	+	+
UM9	+	+	+	+
176	+	+	+	+
177	+	+	+	+
162		+		+

E.5A: Summary of **A:** The rates at which UK MRSA interacted with the KB skin and A549 lung mammalian cell lines; and **B:** The cytotoxic effects of live UK MRSA on both KB skin and A549 lung mammalian cell lines. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed unpaired t-test. Higher means and significant *P* are in purple letters and red letters respectively

A

MOI	UK Means Compared (%)		*P*-value
	KB	A549	
Association			
10:1	0.038	0.032	0.404
100:1	0.0254	0.0178	0.046
Invasion			
10:1	0.021	0.0144	0.230
100:1	0.0109	0.0081	0.294
Total Survival			
10:1	47.217	62.85	0.388
100:1	5.095	7.49	0.247

B

MOI	UK Means of the cytotoxic effects Compared (%)		*P*-value
	KB	A549	
6 hrs			
10:1	2.12	1.17	0.084
100:1	0.9	3.06	0.012
24hrs			
10:1	15.24	11.02	0.437
100:1	31.34	21.11	0.191

E.5B: Mean numbers of intracellular bacteria recovered following invasion of KB and A549 cells by UK MRSA

MOI	Mean number of intracellular bacteria recovered following invasion (CFU)	
	UK MRSA	
	KB	A549
10:1	207222	144444
100:1	1094444	810000

E.6A: Summary of **A:** The rates at which Nigerian MRSA interacted with the KB skin and A549 lung mammalian cell lines; and **B:** The cytotoxic effects of live Nigerian MRSA on both KB skin and A549 lung mammalian cell lines. Values of $P < 0.05$ were accepted as significantly different based on the 2-tailed unpaired t-test. Higher means and significant *P* are in purple letters and red letters respectively

A

MOI	Nigerian Means Compared (%)		*P*-value
	KB	A549	
Association			
10:1	0.028	0.029	0.885
100:1	0.0240	0.0164	0.059
Invasion			
10:1	0.023	0.014438	0.066
100:1	0.0111	0.0092	0.388
Total Survival			
10:1	60.729	76.65	0.215
100:1	7.475	11.19	0.081

B

MOI	Nigerian Means of the cytotoxic effects compared (%)		*P*-value
	KB	A549	
6 hrs			
10:1	4.42	4.19	0.987
100:1	4.15	8.65	0.009
24 hrs			
10:1	45.97	31.73	0.199
100:1	57.02	32.00	0.083

E.6B: Mean numbers of intracellular bacteria recovered following invasion of KB and A549 cells by UK MRSA

MOI	Mean number of intracellular bacteria recovered following invasion (CFU)	
	Nigerian MRSA	
	KB	A549
10:1	232500	144375
100:1	1105417	922708

APPENDIX F: Critical Values of the Mann-Whitney U test

Critical values of U for a Mann–Whitney independent groups test, where U = the smaller of the two possible values and N_a and N_b = the numbers of participants in the two groups. (U is significant if it is less than or equal to the table value)

	N_b															
N_a	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
$p \leq 0.05$ (one-tailed), $p \leq 0.10$ (two-tailed)																
5	4	5	6	8	9	11	12	13	15	16	18	19	20	22	23	25
6	5	7	8	10	12	14	16	17	19	21	23	25	26	28	30	32
7	6	8	11	13	15	17	19	21	24	26	28	30	33	35	37	39
8	8	10	13	15	18	20	23	26	28	31	33	36	39	41	44	47
9	9	12	15	18	21	24	27	30	33	36	39	42	45	48	51	54
10	11	14	17	20	24	27	31	34	37	41	44	48	51	55	58	62
11	12	16	19	23	27	31	34	38	42	46	50	54	57	61	65	69
12	13	17	21	26	30	34	38	42	47	51	55	60	64	68	72	77
13	15	19	24	28	33	37	42	47	51	56	61	65	70	75	82	84
14	16	21	26	31	36	41	46	51	56	61	66	71	77	82	87	92
15	18	23	28	33	39	44	50	55	61	66	72	77	83	88	94	100
16	19	25	30	36	42	48	54	60	65	71	77	83	89	95	101	107
17	20	26	33	39	45	51	57	64	70	77	83	89	96	102	109	115
18	22	28	35	41	48	55	61	68	75	82	88	95	102	109	116	123
19	23	30	37	44	51	58	65	72	80	87	94	101	109	116	123	130
20	25	32	39	47	54	62	69	77	84	92	100	107	115	123	130	138

	N_b															
N_a	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
$p \leq 0.01$ (one-tailed), $p \leq 0.02$ (two-tailed)																
5	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
6	2	3	4	6	7	8	9	11	12	13	15	16	18	19	20	22
7	3	4	6	7	9	11	12	14	16	17	19	21	23	24	26	28
8	4	6	7	9	11	13	15	17	20	22	24	26	28	30	32	34
9	5	7	9	11	14	16	18	21	23	26	28	31	33	36	38	40
10	6	8	11	13	16	19	22	24	27	30	33	36	38	41	44	47
11	7	9	12	15	18	22	25	28	31	34	37	41	44	47	50	53
12	8	11	14	17	21	24	28	31	35	38	42	46	49	53	56	60
13	9	12	16	20	23	27	31	35	39	43	47	51	55	59	63	67
14	10	13	17	22	26	30	34	38	43	47	51	56	60	65	69	73
15	11	15	19	24	28	33	37	42	47	51	56	61	66	70	75	80
16	12	16	21	26	31	36	41	46	51	56	61	66	71	76	82	87
17	13	18	23	28	33	38	44	49	55	60	66	71	77	82	88	93
18	14	19	24	30	36	41	47	53	59	65	70	76	82	88	94	100
19	15	20	26	32	38	44	50	56	63	69	75	82	88	94	101	107
20	16	22	28	34	40	47	53	60	67	73	80	87	93	100	107	114

http://www.psychologywizard.net/mann-whitney-u-test-ao1-ao2.html

Publications and Presentations

Publication

Li, J., Olaleye, E., Kong, X., Zhou, T., Ma, Y., Jurach, J., Al Rugaie, O., Hider, R. C., Zhang, G., Alsam, S. and Abbate, V. (2016) 'Macromolecular iron-chelators via RAFT-polymerization for the inhibition of methicillin-resistant *Staphylococcus aureus* growth', *Polymer*, 87, 64-72

Oral Presentation

2017

Olaleye E. D. and Alsam S.

A study on the differences between the virulence patterns of UK and Nigerian MRSA isolates. 16th Annual Graduate Forum of the School of Biological Sciences, University of Essex

Posters

2015

Olaleye E. D. and Alsam S.

The inhibitory effect of novel iron-chelators on MRSA and their cytotoxic effect on KB epithelial cell line. 14th Annual Graduate Forum of the School of Biological Sciences, University of Essex

2017

Olaleye E. D. and Alsam S.

A study of the differences among the antibiotic susceptibility and virulence patterns of UK and Nigerian MRSA isolates. Microbiology Society Annual Conference