Role of extended spectrum beta lactamases producing *E. coli* and *Acanthamoeba* in urinary tract infections

Alaa Talal Qumsani

A thesis submitted for the degree of Doctor of Philosophy in Microbiology

School of Biological Sciences

University of Essex

January 2019

Abstract

About 150 million cases of Urinary Tract Infections (UTIs) happen annually worldwide costing the NHS billions of pounds. Antibiotic resistance caused by Extended-Spectrum β-lactamases (ESBLs) producing organisms, particularly *E. coli*, have become an increasing problem, and recurrent infections are not uncommon. It has been confirmed that pathogenic bacteria survive and multiply inside the protozoan parasite, *Acanthamoeba*. The indiscriminate use of various antimicrobial drugs has resulted in the development of drug resistance. Therefore, looking for new or additional antimicrobial compounds is urgent.

Four clinical strains of *E. coli* (three ESBL+ve (TEM, AmpC, and OXA-48) and one ESBL–ve used as a control) were used in this project to study the interaction of the above microorganisms with the urinary tract and their possible alternative treatment. All strains were characterised by PCR and sensitivity testing. Results showed that all ESBL producers were sensitive to Ciprofloxacin. OXA-48 displayed an increased resistance when compared with other strains. The 2-D gel electrophoresis was used to further identify the microorganisms and results showed many proteins were featured in OXA-48 compared with others. All uropathogens exhibited the ability to associate with and invade *Acanthamoeba* T4 and the urothelial cell line (TRET-NHUC). However, OXA-48 was the only one survived and multiplied inside both *Acanthamoeba* and the cell line. This project has confirmed that cytotoxicity relies on the number of intracellular bacteria. Furthermore, the cytotoxic effect of bacterial conditioned medium was higher than live and heat-killed bacteria. This study also investigated the antimicrobial efficacy of cetylypyridinium chloride (CPC). An increased concentration of CPC has a positive correlation with the inhibition of bacteria. To confirm the presence of *Acanthamoeba* in urine, 76 samples were collected from patients with ESBL+ve bacteria. It is interesting to report that more than 17% of urine samples were positive for *Acanthamoeba* supporting our hypothesis that the amoeba may play a role in UTIs.

Acknowledgements

First of all, I praise Allaah, the almighty for providing me with the opportunity and granting me the capability to proceed successfully. My deepest gratitude goes to Umm al-Qura University for awarding me a full scholarship to complete my postgraduate studies.

I would like to express my appreciation and sincere gratitude to my supervisor Dr Selwa Alsam, for her academic supervision and personal support throughout all my years in the school. I am particularly indebted to Selwa for her constant faith in my lab work. Without her encouragement and her patience, this PhD would not have been completed.

I am also indebted to Prof. Graham Mitchell, for his valuable suggestions, support and help during our scientific group meetings; and as well for his editorial input for my thesis. Many thanks goes to my committee members, Dr Jordi Paps and Dr Tony Elston for their support and inspiring suggestions during the board meetings.

I am very grateful to my previous and current lab colleagues: Dr Osamah Al-Rugaie, Dr Saleh Al-Ghamdi, Dr Aiden Matthew Jayanth, Dr Bauyrzhan Tashmukhambetov, Miss Eniola Olaleye, and Miss Amna Alotaiba; and lab technicians: Miss Lynwen James, Mrs Julie Arvidson, and Mrs Amanda Clements for their enthusiastic support and help. I would express my best wishes to our postgraduate research administrator, Mrs Emma Revill for her assistance, help and support. I am also indebted to my best friend Nawaf Shujaa for his support and help.

My deepest thanks to my Father Talal and my mother for them continues love, support, encouragements with their best wishes. My thanks to my brother, sisters, Father-in-law, and Mother-in-law for their support, encouragements with their best wishes. Finally, I would like to express my appreciation and thank my wife and my children (Remas, Talal, and Makki), for their constant love and continuous encouragement and patience.

Contents

Abstract....II
Acknowledgements....III
Contents....IV
List of Figures....IX
List of Tables....XIII
List of Abbreviations....XV
1. Introduction....1
1.1 Urinary tract....2
1.1.1 Background....2
1.1.2 Anatomy of the urinary system....4
1.1.3 Urinary tract infections....4
1.1.4 Microorganisms associated with UTIs....6
1.2 *E. coli*....10
1.2.1 Background....10
1.2.2 Cell structure of *E. coli*....11
1.2.3 Bacterial virulence factors....13
1.2.4 Antibiotic resistance....16
1.2.5 Bacterial interaction and invasion of cells....30
1.3 *Acanthamoeba*....35
1.3.1 Background....35
1.3.2 Ecology....35
1.3.3 Life Cycle....36
1.3.4 Feeding....37
1.3.5 Classification of *Acanthamoeba*....38
1.3.6 *Acanthamoeba* human infections....38
1.3.7 *Acanthamoeba* pathogenesis....42
1.3.8 Bacterial correlation with *Acanthamoeba*....44
1.4 Current treatment....46
1.4.1 Alternative therapeutic techniques....47
1.4.2 Cetylpyridinium Chloride (CPC)....48
1.5 Aims and objectives....50
2. Characterisation of *E. coli* multi-drug resistant bacteria (TEM, AmpC, and OXA-48)....52
2.1 Introduction....53

2.2 Materials and methods....57
2.2.1 Bacterial culture....57
2.2.2 Bacterial DNA extraction....57
2.2.3 Bacterial plasmid detaching....58
2.2.4 Bacterial protein extraction....59
2.2.4.1 Bacterial extracellular protein extraction....59
2.2.4.2 Bacterial intracellular protein extraction....59
2.2.5 Genomic analysis....60
2.2.5.1 Polymerase Chain Reaction (PCR)....60
2.2.5.2 Purification of PCR product....63
2.2.5.3 Gene sequencing....64
2.2.5.4 BLAST search and phylogenetic analysis....64
2.2.6 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)....65
2.2.7 Two-Dimensional Gel Electrophoresis (2D Gel)....66
2.2.7.1 First dimensional gel isoelectric focusing (IEF)....66
2.2.7.2 Second SDS-PAGE gel preparation....66
2.2.8 Susceptibility testing by disc diffusion (EUCAST Method)....67
2.2.9 Susceptibility testing by minimum inhibitory concentration (MIC) (E-Test)....68
2.3 Results....69
2.3.1 Polymerase Chain Reaction (PCR)....69
2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)....74
2.3.3 Two-Dimensional Gel Electrophoresis (2D Gel)....75
2.3.3.1 Extracellular protein extraction....75
2.3.3.2 Intracellular protein extraction....83
2.3.4 Susceptibility testing by the disc diffusion method....90
2.3.5 Susceptibility testing by minimum inhibitory concentration (MIC) (E- Test)....95
2.4 Discussion....97
3. Interaction of the uropathogenic bacteria with *Acanthamoeba* and urothelial cell line....102
3.1 Introduction....103
3.2 Materials and methods....107
3.2.1 Bacterial culture....107
3.2.2 *Acanthamoeba* culture....107
3.2.3 Cell line and cell culture....107
3.2.4 Artificial Urine Medium (AUM)....108
3.2.5 Interaction assays....109
3.2.5.1 Association assays....110

3.2.5.2 Invasion assays 110
3.2.5.3 Intracellular survival assays 111
3.2.5.4 Statistical analysis 112
3.2.6 Polymerase Chain Reaction 112
3.2.6.1 DNA extraction 112
3.2.6.2 Polymerase Chain Reaction 113
3.3 Results 115
3.3.1 Association assays of *Acanthamoeba* T4 115
3.3.2 Association assays of TRET-NHUC line 116
3.3.3 Invasion assays of *Acanthamoeba castellanii* 117
3.3.4 Invasion assays of TRET-NHUC line 118
3.3.5 Intracellular survival assays of *Acanthamoeba castellanii* 119
3.3.6 Intracellular bacterial survival assays in TRET-NHUC line 120
3.3.9 Polymerase Chain Reaction (PCR) 121
3.4 Discussion 124
4.1 Introduction 131
4.2 Materials and methods 136
4.2.1 Cell line and cell culture 136
4.2.2 Bacterial culture 136
4.2.2.1 Heat-killed bacteria 136
4.2.2.2 Bacterial Conditioned Media (BCM) 137
4.2.3 *Acanthamoeba* culture 137
4.2.3.1 *Acanthamoeba* Conditioned Medium (ACM) 137
4.2.4 Programmed Cell Death (PCD) assays by flow cytometry 138
4.2.5 Cytotoxicity assays by lactate dehydrogenase (LDH) 139
4.2.6 Software and statistics 140
4.2.7 Genomic analysis 140
4.2.7.1 DNA extraction 140
4.2.7.2 Identification of virulence determinants (toxins and capsule) by PCR 140
4.3 Results 143
4.3.1 Programmed Cell Death (PCD) assays by flow cytometry 143
4.3.1.1 Programmed Cell Death (PCD) assays on TRET-NHUC line exposed to live bacteria 143
4.3.1.2 Programmed Cell Death (PCD) assays for TRET-NHUC line exposed heat-killed bacteria 147
4.3.1.3 Programmed Cell Death (PCD) assays for the CMB on TRET-NHUC line 151
4.3.1.4 Programmed Cell Death (PCD) assays of ACM On TRET-NHUC line 155
4.3.2 LDH assays 158

4.3.2.1 Cytotoxic effects of live bacteria on TRET-NHUC line....158
4.3.2.2 Cytotoxic effects of heat-killed bacteria on TRET-NHUC line....159
4.3.2.3 Cytotoxic effects of the BCM on the TRET-NHUC line....160
4.3.2.4 Cytotoxic effects of ACM on the TRET-NHUC line....161
4.3.3 Polymerase Chain Reaction (PCR)....162
4.4 Discussion....165
5. The effects of cetylpyridinium chloride (CPC) on the uropathogenic bacteria, *Acanthamoeba*, and the urothelial cell line....171
5.1 Introduction....172
5.2 Materials and methods....174
5.2.1 Cell line and cell culture....174
5.2.2 Bacterial culture....174
5.2.3 *Acanthamoeba* culture....174
5.2.4 Artificial Urine Medium (AUM)....174
5.2.5 CPC as an antimicrobial agent....175
5.2.5.1 CPC as an antibacterial agent....175
5.2.5.2 CPC as antiamoebic agent....175
5.2.6 Effects of CPC on intracellular bacteria....176
5.2.6.1 Effects of CPC on intracellular bacteria inside *Acanthamoeba*....176
5.2.6.2 Effects of CPC on intracellular bacteria inside urothelial cell line....176
5.2.7 Cytotoxicity assays by LDH....176
5.2.8 Software and statistics....177
5.3 Results....178
5.3.1 CPC as an antimicrobial agent....178
5.3.1.1 CPC as an antibacterial agent....178
5.3.1.2 CPC as antiamoebic agent....182
5.3.2 Effects of CPC on intracellular bacteria....183
5.3.2.1 Effects of CPC on intracellular bacteria in *Acanthamoeba*....183
5.3.2.2 Effects of CPC on intracellular bacteria in urothelial cell line....186
5.3.3 Cytotoxicity assays by LDH....189
5.4 Discussion....191
6. Presence of *Acanthamoeba* in urine samples from patients with ESBL positive bacteria....195
6.1 Introduction....196
6.2 Materials and methods....199
6.2.1 Ethical approval....199
6.2.2 Collection and processing of urine samples....199

6.2.**3** Preparation of *E. coli* food source stock 200
6.2.4 *Acanthamoeba* culture 200
6.2.4.1 Xenic culture 200
6.2.4.2 Axenic culture 200
6.2.5 Genomic analysis 201
6.2.5.1 DNA extraction 201
6.2.5.2 Polymerase Chain Reaction 202
6.2.5.3 Purification of PCR product 202
6.2.5.4 Gene sequencing 203
6.2.5.5 BLAST search and phylogenetic analysis 203
6.3 Results 204
6.4 Discussion 210
7. General discussion 213
7.1 Overview 214
7.1.1 Antibacterial resistance 214
7.1.2 Recurrent UTIs 216
7.1.3 Possible role of *Acanthamoeba* in UTIs 217
7.1.4 Alternative efficient treatment strategy 219
7.2 Conclusions 221
7.3 Recommendations for future work 221
Appendices 224
Appendix one: Microorganisms and cell line 225
Appendix two: Media 228
Appendix three: PCR and gel electrophoresis 230
Appendix four: Antibiotics, antimicrobial agents, and the other reagents used 236
References 239
Conferences 322

List of Figures

Figure 1.1 a: The male urinary and reproductive tract. b: The female reproductive and urinary tract……..3

Figure 1.2 The variation in the percentage of bacteria causing UTIs……..9

Figure 1.3 Microbial causes of UTIs……....10

Figure 1.4 The structure of the cell wall for gram-negative bacteria……...12

Figure 1.5 Core structure of penicillins, and β-lactam ring in red……19

Figure 1.6 Micrographs showing the life cycle stages of *Acanthamoeba spp*……...37

Figure 1.7 Cornea infected by *Acanthamoeba* keratitis……...……..40

Figure 1.8 Brain imaging by MRI for a patient with granulomatous amoebic encephalitis caused by *Acanthamoeba*.……41

Figure 1.9 The chemical structure of cetylpyridinium chloride (CPC)……..……...49

Figure 2.1: PCR products amplified using 16S rRNA primer……..….70

Figure 2.2: PCR products amplified using TEM primer……...70

Figure 2.3: PCR products amplified using an OXA-48 primer.……71

Figure 2.4: PCR products amplified using CTX M primer……...…71

Figure 2.5: PCR products amplified using Usp primer……..…72

Figure 2.6: The phylogenetic tree of comparison of all samples (TEM, AmpC, and OXA-48) and the three positive controls……..…72

Figure 2.7: The AmpC strain with 16S rRNA primer,……..73

Figure 2.8: The AmpC strain with AmpC primer……..74

Figure 2.9: Protein profile of *E.coli* strains by SDS-PAGE……..…75

Figure 2.10: Representation of a reference image showing all differentially expressed extracellular proteins of *E. coli* (ESBL-ve).…….……...76

Figure 2.11: Representation of reference image showing all differentially expressed spots after analysis extracellular of proteins for *E. coli* (ESBL-ve)……..……..……77

Figure 2.12: The 3D representations of spots identified by the Progenesis SameSpot software…….……..…82

Figure 2.13: Representation of reference image showing all differentially expressed spot after analysis intracellular protein for *E. coli* (ESBL-ve).…….84

Figure 2.14: Representation of a reference image showing all differentially expressed intracellular proteins of *E. coli* (ESBL-ve)…….…...85

Figure 2.15: The 3D representations of spots identified by the Progenesis SameSpot software..86

Figure 2.16: TEM strain showing resistance to antibiotics compared with ESBL –ve strain…..92

Figure 2.17: AmpC strain showing resistance to antibiotics compared with ESBL –ve strain...93

Figure 2.18: OX-48 strain is showing resistant to antibiotics compared with ESBL –ve a strain…….…...94

Figure 2.19: The MIC of Augmentin against ESBL +ve strains compared with the control ESBL–ve strain…….……..96

Figure 3.1 Steps of bacterial interaction assays (association, invasion and survival) with *Acanthamoeba*……..……..112

Figure 3.2 The percentage of bacteria associated per *Acanthamoeba* T4……..116

Figure 3.3 The percentage of bacteria associated per TRET-NHUC line……..117

Figure 3.4 The percentage of invaded bacteria per *Acanthamoeba T4*……..118

Figure 3.5 The percentage of invaded bacteria per TRET-NHUC line……..119

Figure 3.6 The percentage of bacteria survive inside *Acanthamoeba T4*……..120

Figure 3.7 The percentage of bacteria surviving inside TRET-NHUC line……..121

Figure 3.8: PCR products amplified using PapA, PapC, FimH, Afa/deaBC, and Iss primers...123

Figure 4.1: Cytotoxic effects of live strains of *E. coli* (ESBL+ve and ESBL–ve)…….…….145

Figure 4.2: Cytotoxic effects of 10 bacteria live/cell on TERT-NHUC line……..146

Figure 4.3: Cytotoxic effects of 100 bacteria live/cell on TERT-NHUC line……....146

Figure 4.4: Cytotoxic effects of heat-killed bacteria strains of *E. coli* (ESBL+ve and ESBL–ve)……149

Figure 4.5: Cytotoxic effects of 10 heat-killed bacteria/cell on TERT-NHUC line…….…...150

Figure 4.6: Cytotoxic effects of 100 heat-killed bacteria/cell on TERT-NHUC line……..150

Figure 4.7: Cytotoxic effects of BCM of *E. coli* (ESBL+ve and ESBL–ve)……...153

Figure 4.8: Cytotoxic effects of BCM of 10 bacteria/cell on TERT-NHUC line……....154

Figure 4.9: Cytotoxic effects of BCM of 100 bacteria/cell on TERT-NHUC line……..154

Figure 4.10: Cytotoxic effects of ACM…….…….…….…156

Figure 4.11: Cytotoxic effects of ACM at 10µl on TERT-NHUC line……...157

Figure 4.12: Cytotoxic effects of ACM at 100µl on TERT-NHUC line…….157

Figure 4.13 Cytotoxic effects of live bacteria on TRET-NHUC line at 10, 100 bacteria/cell after 24h……...159

Figure 4.14 Cytotoxic effects of heat-killed bacteria on TRET-NHUC line at 10, 100 bacteria/cell doses after 24h…….……160

Figure 4.15 Cytotoxic effects of the BCM on the TRET-NHUC line at 10 and 100 bacteria/cell doses after 24h……..…161

Figure 4.16 Cytotoxic effects of the ACM on the TRET-NHUC line at 10µl and 100µl after 24h…….……..162

Figure 4.17: PCR products amplified using hlyD, CNF-1, pic, sat, and kbsII (Kii) primers….164

Figure 5.1 Effect of CPC (1.5µg/ml) on the growth of uropathogenic bacteria in AUM……..179

Figure 5.2 Effect of CPC (3µg/ml) on the growth of uropathogenic bacteria in AUM…..……180

Figure 5.3 Effect of CPC (5µg/ml) on the growth of uropathogenic bacteria in AUM……..…181

Figure 5.4 Effect of CPC (10µg/ml) on the growth of uropathogenic bacteria in AUM……...182

Figure 5.5 The cytotoxicity effect of CPC on *Acanthamoeba* (T4)…….……..183

Figure 5.6 Effect of different concentrations of CPC on intra-amoebic uropathogenic bacteria (OXA-48 strain at 10 bacteria/amoeba) in *Acanthamoeba* (T4)……185

Figure 5.7 Effect of different concentrations of CPC on intra-amoebic uropathogenic bacteria (OXA-48 at 100 bacteria/amoeba) in *Acanthamoeba* (T4)…….186

Figure 5.8 Effect of different concentrations of CPC on intracellular of uropathogens bacteria (OXA-48 at 10 bacteria/cell) in TERT-NHUC line…….……188

Figure 5.9 Effect of different concentrations of CPC on intracellular of uropathogens bacteria (OXA-48 at 100 bacteria/cell) in TERT-NHUC line…….……..189

Figure 5.10 CPC induced TERT-NHUC line cytotoxicity…….……..…190

Figure 6.1: Presence of *Acanthamoeba spp.* in the positive urine samples…….206

Figure 6.2: The percentage of displaying the prevalence of *Acanthamoeba* in urine among female and male……...207

Figure 6.3: Pie chart is displaying the spread of different species in *Acanthamoeba* positive urine samples…….…207

Figure 6.4: PCR products amplified using 16s rRNA primer……..208

Figure 6.5: 16s rRNA phylogenetic trees for the 13 positive samples and two reference sequences…….209

List of Tables

Table 1.1 Most common microorganisms grown in urine cultures……..……..7

Table 1.2: Most common microbes causing UTIs in Shahrekord……..….8

Table 1.3: Timeline is showing the use of various classes of antibiotics and describing resistance to that class of antibiotic in formerly susceptible microbes……...22

Table 1.4 Classification of β-lactamases according to Ambler molecular scheme……...24

Table 2.1 Gene and primer sequences for the phylogenetic grouping of *E. coli*……...61

Table 2.2 The setup of PCR cycling for the different genes under investigation……..……62

Table 2.3 The Forty-eight specific protein spots identified by pI and MW among the clinical strains……78

Table 2.4 the spot numbers identified with the Progenesis Same Spot software……..79

Table 2.5 The Forty-four specific protein spots identified by pI and MW between the clinical strains……87

Table 2.6 the spot numbers identified with the Progenesis Same Spot software……..…88

Table 2.7: susceptibility testing of ESBL +ve and –ve strains of *E. coli* against different antibiotics……...91

Table 2.8: Susceptibility testing of ESBL +ve and –ve strains of *E. coli* against amoxicillin/clavulanic acid antibiotic……....95

Table 3.1: AUM composition…….….109

Table 3.2 Gene and primer sequences for the virulence factors of *E. coli*……..113

Table 3.3 The setup of PCR cycling for the different genes under investigation…….……...114

Table 3.4: Summary of virulence factors genes amplified from the genomic DNA of the strains under study (ESBL-ve and ESBL +ve)……....122

Table 4.1 Gene and primer sequences for the virulence factors of *E. coli*……..141

Table 4.2 The setup of PCR cycling for the different genes under investigation……....142

Table 4.3: Summary of virulence factors amplified from the genomic DNA of the strains under study (ESBL-ve and ESBL+ve)……...…163

Table 6.1 Gene and primer sequences for the *Acanthamoeba* under investigation…….202

Table 6.2 The setup of PCR cycling for the *Acanthamoeba* 16S rRNA under investigation…..202

Table 6.3: List of urine samples from patients with ESBL+ve and tested positive for *Acanthamoeba*……205

List of Abbreviations

(μ, m)M	(micro, milli) Molar
(μ, m, k)g	(micro, milli, kilo) gram
°C	Celsius
18s rRNA	18S ribosomal Ribonucleic acid
2D Gel	Two-dimensional gel electrophoresis
A. castellanii	*Acanthamoeba castellanii*
Abs	Antibacterial biomaterials
ACM	*Acanthamoeba* conditioned medium
ADP	Adenosine diphosphate
AGE	*Acanthamoeba* Granulomatous Encephalitis
AK	*Acanthamoeba* Keratitis
ATP	Adenosine triphosphate
AUM	Artificial Urine Medium
BCM	Bacteria Conditioned Medium
BLAST	Basic Alignment Search Tool
BSAC	British Society for Antimicrobial Chemotherapy
CD	Clinical diagnosis
CDC	Centre for Disease Control
CFU	Colony forming units
CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate
CHUFT	Colchester Hospital University Foundation Trust
CLED	Cysteine lactose electrolyte deficient
CM	Conditioned Medium
CNF	Cytotoxic necrotising factors
CO2	Carbon dioxide
CPC	Cetylypyridinium chloride
CTXM	Cefotaximases-type
DI	Disseminated infection
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	2’-deoxynucleoside-5’-triphosphates

DTT	Dithiothrietol
dw	distilled sterile water
E.coli	*Escherichia coli*
ECDC	The European Centre for Disease Control and Prevention
ESBL	Extended Spectrum β-Lactamase
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FITC	Fluorescein isothiocyanate
FLA	Free-living amoeba
FSC	fetal calf serum
h	Hour (Hrs)
HCl	Hydrochloric acid
HGT	Horizontal gene transfer
HlyA	α-haemolysin
IBCs	Intracellular bacterial communities
IEF	Isoelectric focusing
IPG	Immobilized pH-gradient
kDa	kilo Daltons
KGM-2	Keratinocyte growth medium 2
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LPS	Lipopolysaccharide
MBP	Mannose Binding Protein
MH	Mueller Hinton
MIC	Minimum inhibitory concentration
Min	Minute (s)
MW	Molecular Weight
NHS	National health service
NNA	Non-nutrient agar
OD	Optical density
OmpA	Outer membrane protein A
OXA	Oxacillinase-type
pb	Base pair

PBPs	penicillin binding proteins
PBS	Phosphate buffered saline
PCD	Programmed Cell Death
PCR	Polymerase Chain Reaction
pI	Isoelectric point
PLs	Phospholipases
PYG	Peptone yeast extract glucose
PS	Phosphatidylserine
QACs	Quaternary ammonium compounds
RNA	Ribonucleic acid
rRNA	ribosomal ribonucleic acid
RT	Room temperature
rUTIs	Recurrent UTIs
s	Second
SDS PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SHV	Sulphydryl variable
spp	Species
TEM	Temoneira
TEMED	Tetramethylethylenediamine
TRET-NHUC	Telomerase Reverse Transcriptase-immortalised NormalHuman Urothelial Cell
UIN	unique identification number
UK	United Kingdom
UPEC	Uropathogenic *E. coli*
UTIs	Urinary tract infections
UV	Ultra violet light
VFs	Virulence Factors
v/v	Volume for volume
w/w	Weight for weight

CHAPTER ONE 1

1. Introduction

1.1 Urinary tract

1.1.1 Background

Urinary tract infections (UTIs) are the most common bacterial infections in clinical practice and are a global issue because many organisms are associated with them. An estimated 150 million cases of UTIs happen every year around the world, resulting in over £4 billion in healthcare costs (Kucheria *et al.*, 2005; Ali *et al.*, 2009; Beahm *et al.*, 2017; Cruz *et al.*, 2019). Based on Croxall et al. United Kingdom (UK) has an annual rate of UTIs in the elderly population ranging from 10% in the community to as high as 30% of hospitalised patients. Mortality rates in elderly patients from bacteraemia as a result of UTIs can be around 33% (Croxall *et al.*, 2011). A study by Plowman et al. found that over 12 months, UTIs had the highest incidence (35%) of all nosocomial infections in a district general hospital in the UK, affecting patients over 60 years of age (Plowman *et al.*, 2001). UTIs are the most common bacterial infection treated with antibiotics in long-term care facilities, where they account for 20–60% of all antibiotic usage (Nicolle, 2001).

Adult females are the most commonly affected group, while other high-risk groups include children, the elderly, people with structural abnormalities of the urinary tract and those having a urinary tract intervention like catheter insertion (Ali *et al.*, 2009). Anatomical differences, hormonal effects and sexual activity influence this pattern of occurrence. UTIs are a more common health problem in women than men because they have a shorter urethra which is closer to the anus and vagina (Figure 1.1) (Griebling, 2005; Tadesse *et al.*, 2014).

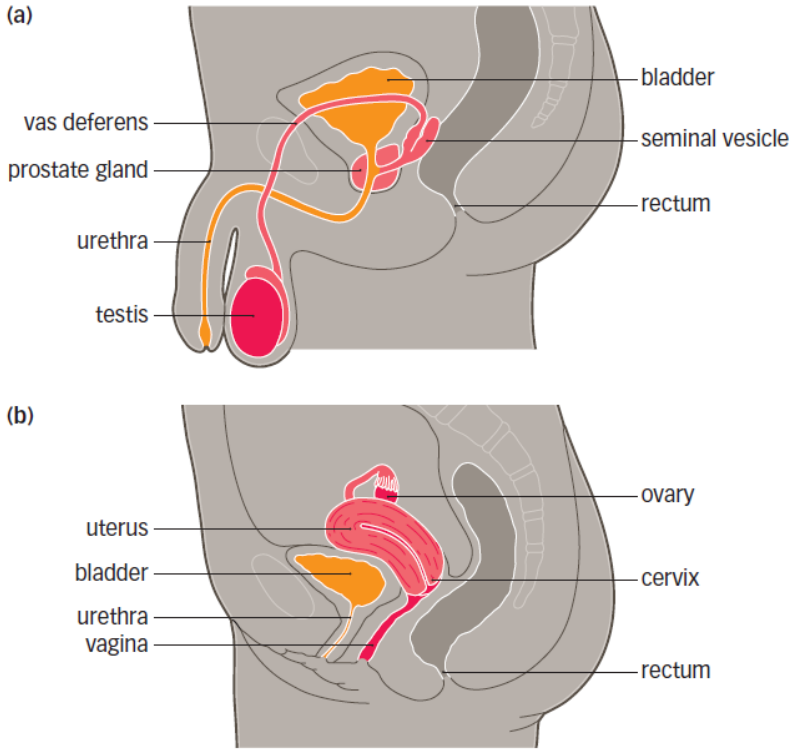

Figure 1.1 a: The male urinary and reproductive tract. b: The female reproductive and urinary tract. In both male and female these tracts are portals of entry for pathogens (Strelkauskas *et al.*, 2010).

Urinary tract infections are more common in women during pregnancy due to changes in the urinary tract. Hormones cause changes in the urinary tract that lead to infections in women. Increases in urinary progestins and estrogens may lead to a decreased of the lower urinary tract to resist invading bacteria. Moreover, a growing uterus presses on the bladder; prevent the complete emptying of urine, so the rest of stagnant urine is a likely source of infection. Untreated, these infections may lead to kidney infections (Delzell *et al.* 2000; Tadesse *et al.* 2014).

There are four types of UTIs based on the organ infected. The first one is urethritis which affects the urethra, the second one is cystitis which occurs in the bladder, the third is called nephritis, which affects the kidneys, and the last one is prostatitis, which affects the prostate (Strelkauskas *et al.*, 2010). Also, UTIs can be classified into two types, depending on underlying

host factors and uropathogens: uncomplicated or complicated (Davis and Hugh 1999; Vitaly Smelov *et al.* 2016).

1.1.2 Anatomy of the urinary system

The urinary system removes body waste from the blood in the form of urine. The urinary system consists of a group of organs include the two kidneys, two ureters, a urinary bladder and the urethras (Figure 1.1) (Lo and Alonto, 2011). Usually, the kidneys are located in the middle of the back one on each side of the spine, and the lower ribs protect some of the parts of the kidney. Each kidney has about 1 million nephrons, which are tiny filters in the kidney. The main function of the kidneys is to filter the blood. Each ureter, about 8 to 10 inches long, connects a kidney to the urinary bladder. The urinary bladder is a hollow organ that sits in the pelvis, and stores urine. Urine formed in the kidney flows to the tube-like ureter where it carries urine to the bladder. The wall of each ureter has smooth muscles which regularly contract and relax to force urine into the bladder. The last part of the urinary system is the urethra, where urine flows out of the body (Lo and Alonto, 2011). The length of the urethra is different between men and women. For men, the urethra measures about 6 to 8 inches long. On the other hand, the female urethra is much shorter, about 1.5 inches. This is one of many reasons why females are prone to having UTIs: the shorter distance that other microorganisms such as bacteria travel going up the urinary tract (Tadesse *et al.*, 2014).

1.1.3 Urinary tract infections

Several different types of agents can occur within the urinary tract to cause UTIs. Early evidence by an epidemiological study comparing nuns and married women proved sex involvement in the aetiology of UTI (Kunin and McCormack, 1968). Another study showed that

in sexually active young women, the incidence of symptomatic UTIs is high (Hooton *et al.*, 1996). Intercourse frequency and spermicide use itself were also found to be associated with an increased risk of UTIs (Scholes *et al.*, 2000). Spermicide alters normal vaginal flora and facilitates colonisation with pathogens (Hooton *et al.*, 1994).

Cystitis is most common during pregnancy because of changes in the urinary tract and uterus (Ali *et al.*, 2018). The most common factors that lead to UTIs in pregnant women are dilation of the ureters and renal pelvises, increased urinary pH, and glycosuria promoting bacterial growth (Care *et al.*, 2005). About 10-30% of women with bacteria in the first trimester develop an upper urinary tract infection in the second or third trimester (Sign, 2006). Lower tract infections are the most common in diabetic people (Geerlings *et al.*, 2002), probably due to the deficiency of local immune mechanisms.

Ageing can be a factor in causing UTIs; this happens by decreasing the action of muscles within the urinary tract and decrease excretion of urine; therefore, urine may back up, and an infection can develop (Lo and Alonto, 2011). Also, Injuries from trauma or surgery can cause infection as well as insertion of urinary catheters (Nicolle, 2001, 2014; Morgan, 2007).

The indiscriminate use of various antimicrobial drugs for infectious disease treatment has resulted in the drug resistance in pathogens in recent years and recurrent UTIs (Ahmed *et al.*, 2017). Re-recurrence of UTIs is more common, with 27% of patients suffering another infection within six months and 44% of patients experiencing another infection within one year. Between one-third and one-half of the recurrent infections are caused by the same strain as initiated the disease. Thus, the recurrent infections will help the bacteria to become more resistant to antimicrobials (Hilbert, 2013; Glover *et al.*, 2014). Also, the other study confirmed that 65% of recurring UTIs seem to be caused by the same microorganism. Thus, the recurrent infections will help the bacteria to become more resistant to antimicrobials (Hilbert, 2013; Kelley *et al.*, 2014).

The organisms are likely to cause recurrence of infection and become more resistant to antimicrobials after each course of treatment (Pallett and Hand, 2010).

An untreated UTI may spread to the kidney or bladder, causing more pain and illness. It can also cause sepsis. The term urosepsis is usually used to describe sepsis caused by a UTI. Sepsis and septic shock can result from an infection anywhere in the body, such as urinary tract infections. Sometimes incorrectly called blood poisoning, sepsis is the body's often deadly response to infection or injury. Sepsis kills and disables millions and requires early suspicion and rapid treatment for survival. People shouldn't die from a UTI, but if sepsis begins to take over and develops to severe sepsis and then to septic shock, this is exactly what can happen. More than half the cases of urosepsis among older adults are caused by a UTI. Worldwide, one-third of people who develop sepsis die. Some who do survive are left with life-changing effects, such as post-traumatic stress disorder, chronic pain and fatigue, and limb loss/amputation (Al-Badr and Al-Shaikh, 2013).

Finally, the most important agents that cause UTIs are microbes such as bacteria, viruses, and fungi. Bacteria are the most common kind of microorganisms to cause UTIs (Nicolle, 2001).

1.1.4 Microorganisms associated with UTIs

The organisms that cause UTIs are found in many species of gram-negative Enterobacteriaceae related to the lipopolysaccharide (LPS) layer of the gram-negative bacterial cell envelope (Madigan *et al.*, 2009; Brown *et al.*, 2018). These include *Escherichia coli* (*E. coli*) isolated in around 75-90% of cases (Kucheria *et al.*, 2005). The most common pathogen that causes UTIs amongst Enterobacteriaceae (Tables 1.1 and 1.2) (Figure 1.2) is *E. coli* which account for 50% of hospital-acquired and 85% of community-acquired UTIs (Davis and Hugh 1999). Another study found *E. coli* was the most commonly isolated pathogen responsible for

UTIs (138/194 - 71.1%) (Mahesh *et al.*, 2011). Non-pathogenic *E. coli* is an essential part of the normal flora in the human gastrointestinal tract. However, *E. coli* that cause UTIs are classified as uropathogenic *E. coli* (UPEC), and these strains account for 75–90% of uncomplicated UTIs and also can cause bacteraemia, sepsis and meningitis (Ahmed *et al.*, 2014). The most common serogroups of *E. coli* causing UTIs worldwide are O4, O6, O14, O22, O75 and O83 (Foxman, 2010; Li *et al.*, 2010). Other Enterobacteriaceae such as *Proteus sp*. and *Klebsiella sp.* are sometimes isolated and gram-positive cocci such as *Enterococcus faecalis* (Naber *et al.*, 2008).

The patient's age may affect the type of infective bacteria present with *Staphylococcus saprophyticus* now accounting for 10% of UTIs in young females compared to less than 1% in elderly female patients (Davis and Hugh 1999). Extended Spectrum Beta-Lactamase (ESBL) are enzymes that can be carried on bacterial plasmids and produced by different gram-negative bacteria especially, *E. coli* that cause UTIs that confer increased resistance to a large number of antibiotics (Figure 1.3) (Kumar and Khan, 2011; Dhillon and Clark, 2012).

Table 1.1 Most common microorganisms grown in urine cultures (Doğru *et al.*, 2013). The pathogens most responsible for grown in urine cultures are Enterobacteriaceae with a high predominance of *E. coli*.

Microbial Strain	**Female patients (n=1227) (%)**	**Male patients (n=488) (%)**	**Total (n=1715) (%)**
E. coli	799 (65.1%)	240 (49.1%)	1039 (60.6%)
Enterococcus spp.	121 (9.9%)	55 (11.2%)	176 (10.30%)
Klebsiella spp.	84 (6.8%)	41 (8.3%)	125 (7.3%)
Pseudomonas spp.	32 (2.6%)	50 (10.1%)	82 (4.8%)
Streptokoklar	54 (4.4%)	2 (0.4%)	56 (3.3%)
Enterobacter spp.	20 (1.6%)	25 (5.1%)	45 (2.6%)
Candida spp.	36 (2.0%)	8 (1.5%)	44 (2.6%)
Proteus spp.	24 (2.0%)	13 (2.6%)	37 (2.2%)
Citrobacter spp.	16 (1.3%)	18 (3.6%)	34 (2.0%)
Others	41 (3.4%)	36 (7.1%)	77 (4.3%)
Total:	1227 (100%)	488 (100%)	17115 (100%)

Table 1.2: Most common microbes causing UTIs in Shahrekord, Iran (Tajbakhsh *et al.*, 2015). The pathogens most responsible for UTI are Enterobacteriaceae with a high predominance of *E. coli*.

Microbial Strain	Number of Males, %	Number of Females, %	UTI, %
Escherichia coli	17 %	34.7 %	51.07 %
Klebsiella pneumonia	4.76 %	11.56 %	16.32 %
Proteus spp.	3.40 %	7.48 %	10.88 %
Acinetobacter spp.	2.04 %	4.08 %	6.12 %
Entrobacter spp.	1.36 %	4.08 %	5.44 %
Citrobacter spp.	0.68 %	3.4 %	4.08 %
Pseudomona saeruginosa	0.68 %	2.72 %	3.40 %
Providencia spp.	0 %	2.04 %	2.04 %

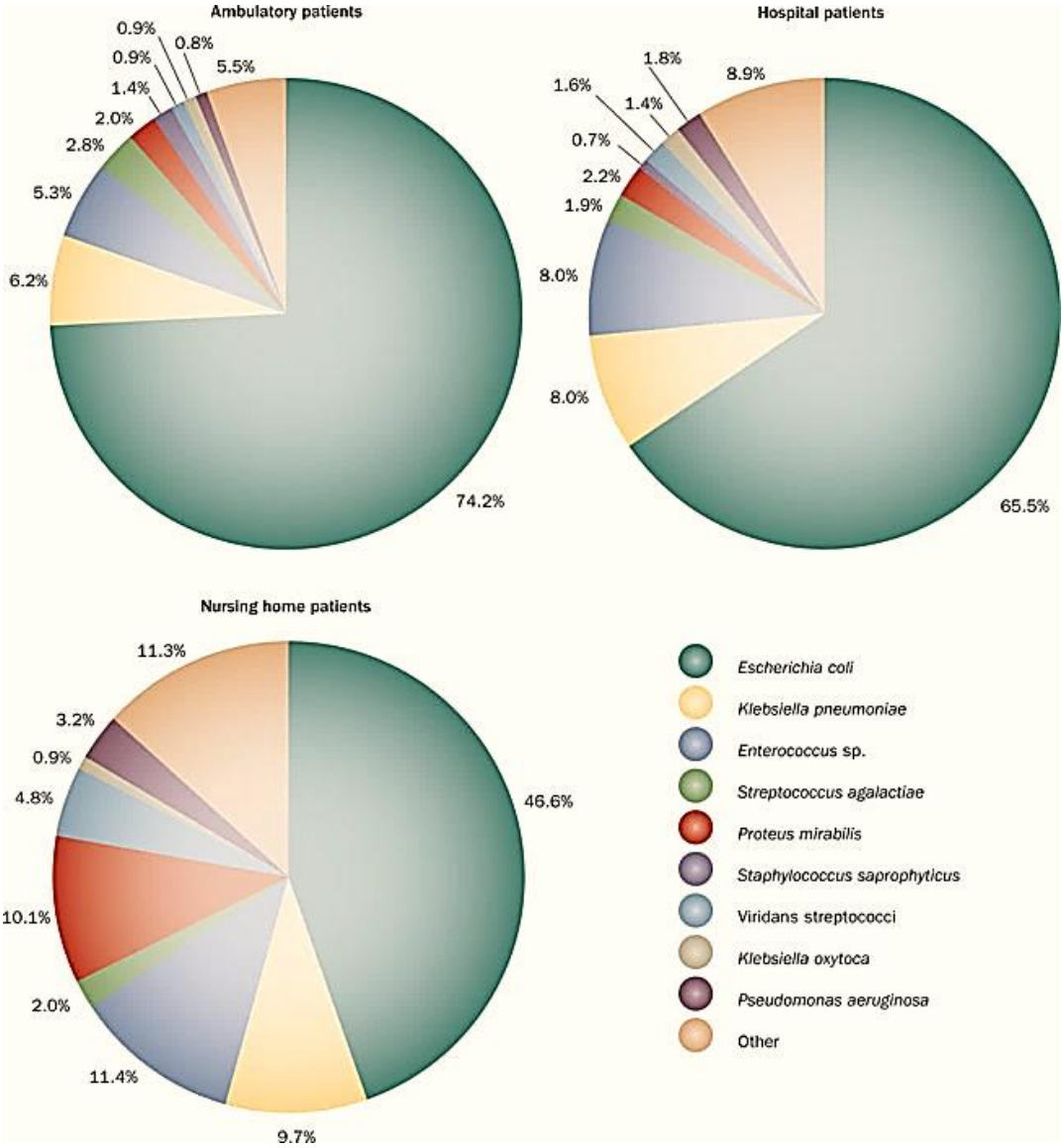

Figure 1.2 The variation in the percentage of bacteria causing UTIs. These results were from a 2004–2005 laboratory-based surveillance study of all community-acquired UTI in the Calgary Health Region of Canada (Foxman, 2010).

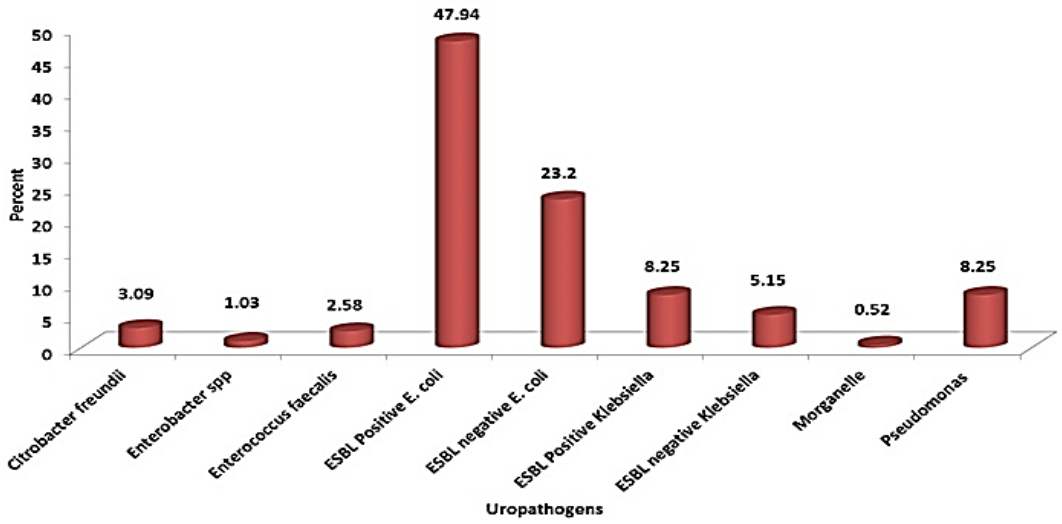

Figure 1.3 Microbial causes of UTIs (Kumar and Khan, 2011). 56.2% of the total infection was caused by ESBL+ve organisms.

1.2 *E. coli*

1.2.1 Background

E. coli was first discovered in 1885 by Theodor Escherich, a German bacteriologist. It is a gram-negative, rod-shaped bacterium; it is one of the common bacteria found in nature and the digestive tract of humans and warm-blooded animals. *E. coli* can survive for long periods in soil, faeces, and water. Usually, *E. coli* serves as an indicator organism for the contamination of water (Samadpour, 1995). It is a facultative anaerobe which uses aerobic respiration if oxygen is present. It is also capable of switching to fermentation or anaerobic respiration if oxygen is absent. The normal growth temperature of *E. coli* is 44°C. However, some laboratory strains can multiply at temperatures of up to 49°C (Fotadar *et al.*, 2005). *E.coli* in large human intestines can help digestion processes, food breakdown and absorption, and vitamin K production (Wilson, 2005).

Many strains of *E. coli* are harmless; however, some can be pathogenic causing intestinal and UTIs in both humans and animals (Jafari *et al.*, 2012). For example, *E. coli* O157: H7, an

epidemiologically significant bacterium, produces a powerful toxin capable of causing the hemolytic-uremic syndrome. *E. coli* O157: H7 can be transmitted to humans by contaminated food, water, and animals (Samadpour, 1995). There are about 190 different serogroups of *E.coli* that are classified based on major surface antigens (Stenutz *et al.*, 2006). The first one is the O antigen that is part of lipopolysaccharide layer; the second one is H antigen, referred to as flagellin; the third one is K antigen that is part of the capsule (Orskov *et al.*, 1977). UPEC is one of the most common causes of UTIs. It is part of the normal flora in the digestive tract and can be introduced in many ways to the urinary tract (Hilbert, 2013; Karam *et al.*, 2018; Malekzadegan *et al.*, 2018).

1.2.2 Cell structure of *E. coli*

E. coli has adhesive fimbriae and a cell wall which consists of an outer membrane containing LPS, a periplasmic space with a peptidoglycan layer and an inner, cytoplasmic membrane. *E. coli* cell walls are strong and tough enough to endure extreme temperatures and pH (Beveridge, 1999; Sekiguchi and Yamamoto, 2012). LPS has different parts: The O antigen, core oligosaccharide, and Lipid A (Figure. 1.4) (Lolis and Bucala, 2003).

The O antigen is a repetitive glycan polymer within the LPS. The O antigen is attached to the core oligosaccharide. Moreover, there are different types of LPS based on O antigen. For example, in *E. coli* there are over 160 O antigen structures produced by different *E.coli* strains (Raetz and Whitfield, 2002). Also, there are two types of LPS based on the presence or absence of O antigen chains, the first one is rough, and the other one is smooth. If the O antigen chains were present, it would make the LPS smooth. In contrast, the absence of O antigen chains would make the LPS rough (Rittig *et al.*, 2003). The rough LPS bacteria usually are more hydrophobic.

Therefore the bacteria with this type of LPS are more sensitive to hydrophobic antibiotics such as gentamicin, kanamycin, and rifamycins (Tsujimoto *et al.*, 1999; Delcour, 2009).

The Core part of LPS contains an oligosaccharide component, and this part links to lipid A. Normally, the core contains sugars such as heptose and 3-deoxy-D-mannooctulosonic Acid (KDO, keto-deoxyoctulosonate). Also, the LPS Cores contain phosphate, amino acids, and ethanolamine substituents (Hershberger and Binkley, 1968; Klein *et al.*, 2013).

The last part of LPS is lipid A, consisting of a phosphorylated glucosamine disaccharide and multiple fatty acids. The primary function of multiple fatty acids chains in lipid A is to stick the LPS into the bacterial membrane. The fatty acid chains are attractive to hydrophobic antibiotics Figure 1.4 (Tzeng *et al.*, 2002; Steimle *et al.*, 2016).

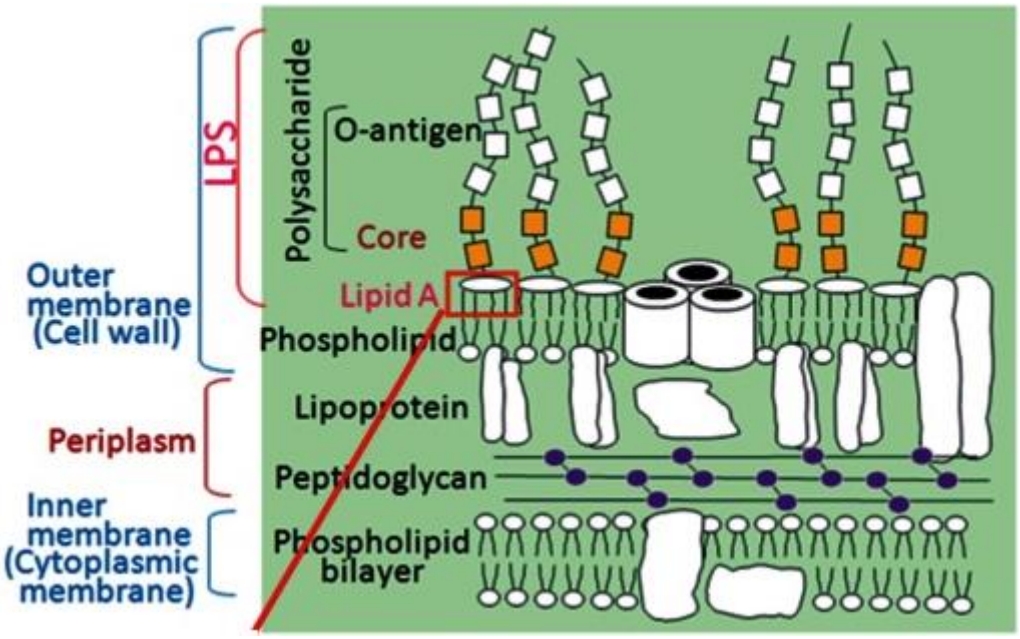

Figure 1.4 The structure of the cell wall for gram-negative bacteria (Matsuura, 2013).

1.2.3 Bacterial virulence factors

The UTIs are the result of the interaction between the host and pathogens. *E. coli* strains are highly adaptable encoding many virulence factors that can enable the bacteria to colonise, infect the urinary tract, and then lead to UTIs. The most common virulence factors of *E. coli* are bacterial capsule, fimbriae, and toxins (Bien *et al.*, 2012; Pereira *et al.*, 2016). Virulence factors of *E. coli* may be divided into two groups contact-dependent, and contact-independent (Davis and Hugh, 1999).

1.2.3.1 Contact-dependent virulence factors

Contact-dependent virulence factors are the presentation of cell surface adhesive organelles in *E. coli* which are the most significant determinants of pathogenicity. *E. coli* may express several adhesions that allow it to attach to urinary tract tissues. The best-characterised type of adhesion is the fimbriae (Mulvey, 2002). Adherence of the bacteria is dependent on three critical environmental characteristics: the bacteria's contact-dependent characteristics, the receptive features of the urothelium, and the fluid that is present between both surfaces (Davis and Hugh, 1999).

Fimbriae are composed of protein subunits and associated with the bacterial surface. The function of fimbriae is to bind the glycolipid and glycoprotein on urothelial cells in the bladder to infect the urinary tract. The most common types of fimbriae in *E. coli* is type 1 fimbriae, type P fimbriae, and type Dr fimbriae (Bien *et al.*, 2012).

First: Type 1 fimbriae:

Type 1 fimbriae are the most common fimbriae on *E. coli*, and this type was produced in more than 80% of pathogenic *E. coli* that cause UTIs. Type 1 fimbriae are composed of a helical rod with repeating FimA subunits that are bound to a distal tip structure containing the FimH

adhesin (Kisiela *et al.*, 2015). Also, type 1 fimbriae bind to the urothelial cells on the adhesin subunit FimH that is located at the fimbrial tip. Moreover, the type 1 fimbriae are responsible for increasing the inflammatory response of bacterial invasion with urothelial cells (Tchesnokova *et al.*, 2011).

Second: Type P fimbriae:

The next most common virulence factor of pathogenic *E. coli* that cause UTIs are type P fimbriae. These fimbriae are believed to play a role in ascending UTIs (Lillington *et al.*, 2014). The attachment of P fimbriae leads to the release of ceramide, which acts as an agonist of Toll-like receptor 4 (TLR4), a receptor involved in activation of the innate immune response including cytokines and antimicrobial peptides; these interactions cause the pain of UTIs (Fischer *et al.*, 2007). In addition, type P fimbriae are very important in the pathogenesis of UTIs especially pyelonephritis. Also, P fimbriae have been implicated as one of the virulence factors involved in acute kidney dysfunction within renal transplant patients (Rice *et al.*, 2006).

Third: Type Dr fimbriae:

One of the most common virulence factors of pathogenic *E. coli* that cause UTIs is type Dr fimbriae (Bien *et al.*, 2012). Fimbriae Dr adhesins of *E. coli* are associated with UTIs, particularly recurrent infection and infection during pregnancy (Delzell *et al.* 2000; Tadesse *et al.* 2014).

1.2.3.2 Contact-independent virulence factors

While much attention is focused on the contact-dependent virulence factors, as many are relatively specific to *E. coli*, like other gram-negative bacteria, there are many virulence factors

located on the *E. coli* such as capsules and flagella. The most common contact-independent virulence factors notably toxins that further enhance its pathogenicity (Strelkauskas *et al.*, 2010).

Lipopolysaccharides (LPS) can also act as a virulence factor to cause pathogenicity. LPS is a core component of the cell wall of gram-negative bacteria. The LPS of *E. coli* is essential in the activation of a proinflammatory cytokine which promotes a systemic inflammation response in UTIs by the induction of nitric oxide as well as cytokine production (Bäckhed *et al.*, 2001).

The next most common non- contact-dependent virulence factor of pathogenic *E. coli* that cause UTIs are flagella. The flagellum is a macromolecular organelle complex that protrudes from the exterior of the bacterial outer membrane up to 15μM long and is responsible for bacterial motility. Typically, an *E. coli* has between four and ten flagella they come together to form a bundle at the posterior pole when swimming. *E. coli* can change direction by one or more motors reversing direction and breaking from the bundle, causing the bacteria to re-orientate (Darnton *et al.*, 2007). The flagella consist of a protein called flagellin, which is in shape helical. It consists of three parts: the motor, hook and filament. The flagella are involved in the interaction of various pathogenic *E. coli* strains with epithelial cells. Flagellated UPEC causes 70–90% of all UTIs, and their pathogenesis involves contact between the bacteria and epithelial cell surface of the urinary tract (Pichon *et al.*, 2009). One study has suggested that *E. coli* flagella have an essential role in the transmission of the bacteria and cause UTIs (Schwan, 2008).

One of the most common contact-independent virulence factors that cause UTIs are toxins secreted by *E. coli*. Production of toxins by colonising *E. coli* may cause an inflammatory response, which leads to symptoms. The most important of these secreted toxins are a lipoprotein called haemolysin, and Cytotoxic Necrotising Factor (CNF) (Bien *et al.*, 2012).

Hemolysins are membrane-damaging toxins that disrupt the plasma membrane of host cells and cause the cells to lyse. These toxins can damage the plasma membrane of both red blood

cells and white blood cells. The most common types of haemolysin are α-hemolysin, and β-hemolysin (Eberspächer *et al.*, 1989). The most significant secreted virulence factor of UPEC is a lipoprotein called α-haemolysin (HlyA), which is frequently associated with UTIs such as pyelonephritis (Johnson, 1991; Vázquez *et al.*, 2017). At high concentrations, it lyses erythrocytes and host cells, leads pathogens to cross epithelial barriers, damage immune cells, and gain access to the host's iron stores (Johnson, 1991). However, at low concentrations, it can induce apoptosis of host immune cells (Smith *et al.*, 2006). The other type of haemolysin that is β-hemolysin has the same interaction of α-hemolysin on the cells (Hsu *et al.*, 2001).

The most common types of CNF are CNF1 and CNF2. CNF has an important function in the regulation of actin cytoskeleton (Kulp and Kuehn, 2010; Strelkauskas *et al.*, 2010). Many studies suggests that CNF1 has effects on the epithelial cell membrane which enhance the ability *E. coli* to invade cells (Bower *et al.*, 2005; Reppin *et al.*, 2017). In addition, the CNF1 has been shown to interfere with polymorphonuclear phagocytosis and cause the death of bladder epithelial cells by apoptosis (Mills *et al.*, 2000). The CNF1 is produced by one-third of all pyelonephritis strains and may also be involved in kidney invasion (Landraud *et al.*, 2000; Ho *et al.*, 2018).

1.2.4 Antibiotic resistance

1.2.4.1 Overview of antibiotic resistance

The development of increased resistance against available antibiotics is a major global concern and is a major threat to the successful treatment of infectious diseases. Many bacteria are developing or have already developed resistance against the currently available antibacterial agents, that includes the development of β-lactams as well (Rawat and Nair, 2010b; Iredell *et al.*, 2016). Antibiotic resistance has spread among bacteria globally because of the exchange of

mobile resistance determinants occurring mostly through transmission of plasmids (Rawat and Nair, 2010b). Several different mechanisms of antibiotic resistance have been evolved via gram-negative bacteria (Iredell *et al.*, 2016). This review is aimed at exploring antibiotic resistance in gram-negative bacteria, mainly the Enterobacteriaceae. These are mostly found to be linked with β-lactamase mediated antibiotic resistance.

1.2.4.2 Mechanisms of antibiotic resistance in gram-negative bacteria

There are different mechanisms for resisting the action of antibiotics. An important mechanism is a modification in the molecule to be targeted by the antibiotic. Penicillin-Binding Proteins (PBPs) serve to be a good example. Besides these, the enzymes involved in the synthesis of peptidoglycan which is responsible for cross-linking the glycan strands to one another and the cell wall of bacteria are also targeted by antibiotics (Heijenoort, 2011; Sauvage and Terrak, 2016).

In some cases, antibiotics are excreted through efflux pumps like Resistance-Nodulation-Division (RND) transporters (Nagano and Nikaido, 2009; Lim and Nikaido, 2010; Li *et al.*, 2015). Another mechanism involves an alteration in the permeability of the membrane through modification of porin molecule (Kojima and Nikaido, 2013; Iredell *et al.*, 2016). Resistance to β-lactams is mostly conferred by enzymatic hydrolysis of the antibiotic, and this mechanism is used by different gram-negative bacteria including *Enterobacteriaceae*. The enzymes responsible for the hydrolysis are β-lactamases like OXA, CTX-M, SHV, TEM and AmpC enzyme families that include ESBLs (Iredell *et al.*, 2016; Xia *et al.*, 2016).

1.2.4.3 Extended-Spectrum Beta-Lactamase (ESBL)

1.2.4.3.1 Background

ESBLs are enzymes that can be carried on bacterial plasmids. They are specific enzymes that can break down many types of antibiotics, which leads to their inactivation. ESBLs cause global health problems since infections caused by enzyme-producing organisms are associated with high morbidity and mortality rates and the financial burden of treatment can be costly. β-lactamases are hydrolytic enzymes which cleave the β- lactam ring conferring bacterial resistance to β-lactam antibiotics, such as penicillins and cephalosporins (Dhillon and Clark, 2012). The β-lactam ring consists of three carbon atoms and one nitrogen atom, as shown in (Figure 1.5). The β-lactam antibiotics usually act by checking the formation of bacterial cell wall as they impede the development of peptide bond between molecules that constitute the peptidoglycan. This impedance is brought about by the antibiotic by targeting the PBPs (Waxman *et al.*, 1980; Fisher *et al.*, 2005). In particular, the β-lactam antibiotics demonstrate irreversible binding with the Ser403 residue of the active site of PBP. This transpeptidation step of peptidoglycan synthesis gets inhibited leading to inhibition of the synthesis of a cell wall of bacteria (Waxman *et al.*, 1980; Silvaggi *et al.*, 2004; Fisher *et al.*, 2005). The abovementioned irreversible binding occurs because β-lactams serve to be analogue of D-alanyl-D-alanine terminal residues of peptidoglycan (Waxman *et al.*, 1980; Silvaggi *et al.*, 2004).

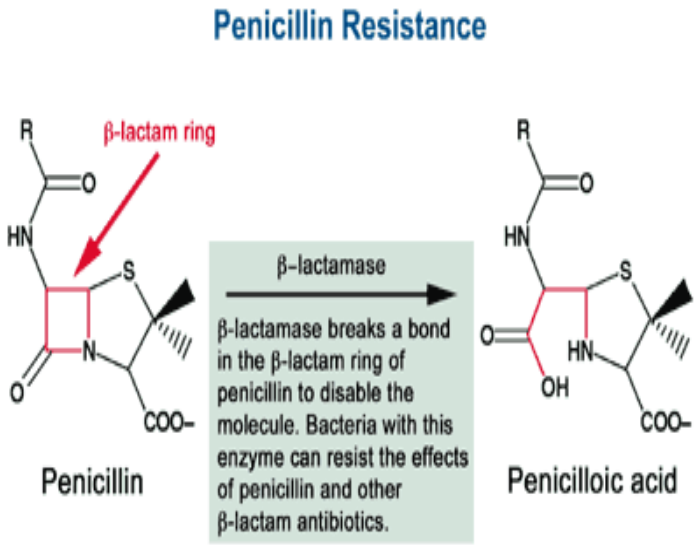

Figure 1.5 Core structure of penicillins, and β-lactam ring in red (Zeng and Lin, 2013).

ESBLs usually are produced by the Enterobacteriaceae family of gram-negative bacteria, especially *Klebsiella pneumoniae* and *E. coli*. They are also produced by other gram-negative bacteria such as *Acinetobacter baumannii*, and *Pseudomonas aeruginosa* (Falagas and Karageorgopoulos, 2009). Since 1980, the beta-lactam antibiotics have been widely used for the treatment of infections, especially by gram-negative bacteria. Resistance against these antibiotic groups developed quickly around the world. In Europe, ESBL-producing Enterobacteriaceae have been spreading, and the rate is increasing dramatically. The first isolates were originally detected in Germany and the UK in 1983 (Mshana *et al.*, 2009; Dhillon and Clark, 2012). In 2011, the European Centre for Disease Control (CDC) and Prevention stated that the spread of ESBLs+ve *E. coli* has increased in more than half of the 28 European countries over the past four years (Beytur *et al.*, 2015).

ESBLs have been described as the β-lactamases which enable bacteria to resist a broad range of β-lactam antibiotics that include penicillins, 1st-, 2nd- and 3rd-generation cephalosporins as well as aztreonam. Yet, cephamycins and carbapenems are excluded from

their resistance profile (Paterson and Bonomo, 2005). Although some enzymes are inhibited by β-lactamase inhibitors like clavulanic acid, the activity of many remains unaffected as well (Paterson and Bonomo, 2005; Bush and Jacoby, 2010).

The first discovered plasmid-encoded ESBL is SHV-2. Its isolation from *K. pneumoniae* was made in 1985 in Germany. It has emerged from a natural point mutation in SHV-1 (Kliebe *et al.*, 1985; Ambler *et al.*, 1991). The discovery of SHV-2 was followed by the discovery of ESBLs emerged from a mutation in blaTEM-1 and blaTEM-2 genes in *Citrobacter freundii*, *E. coli* and *K. pneumoniae* in Germany and France (Sougakoff *et al.*, 1988). Over 170 modified variants of TEM-1 have been discovered since that time, and the majority of these demonstrate the ESBL characteristics (Salverda *et al.*, 2010).

The ESBLs derived from TEM and SHV dominated the period of the 1980s and 1990s. These were often found linked with *K. pneumonia* and hospital-centred outbreaks. In some cases, they were found associated with Enterobacteriaceae like *E. coli* (Bradford, 2001; Paterson and Bonomo, 2005). During the early 2000s, the CTX-M ESBL lineage became prominent because of substantial increment in the evolution rate of CTX-M and spreading of its geographic distribution. These enzymes are not associated with SHV and TEM (Cantón *et al.*, 2012).

The first OXA enzyme derived ESBL was OXA-11. It is a variant of OXA-10 which was discovered in 1991 in Turkey (Hall *et al.*, 1993). Since its discovery, numerous derivatives of OXA-10 have been found. These include OXA-13, OXA-14, OXA-16, OXA-17, OXA-19 and OXA-28. These enzymes have ESBL status (Evans and Amyes, 2014). According to Bush & Jacoby (2010) and Evans & Amyes (2015), certain other derivatives of OXA like OXA-23 and OXA-48 can be of clinical significance because of their association with an increase in resistance to carbapenem.

1.2.4.3.2 Emergence of resistance to β-lactams

Intriguingly, the discovery of AmpC β-lactamase had been made even before the initiation of clinical application of penicillin (Abraham and Chain, 1940; Jacoby, 2009). Resistance to penicillin and other antibiotics emerged during the 1940s and 1950s (Bellamy and Klimek, 1948; Ghuysen, 1991; Livermore and Woodford, 2006; Frère *et al.*, 2016). In the mid-1970s, the inhibitor of β-lactamase, i.e. clavulanic acid was discovered. Thus, the efforts made for overcoming the β-lactamase mediated antibiotic resistance proved fruitful (Brown *et al.*, 1976; Drawz and Bonomo, 2010). After this discovery, synthetic inhibitors like tazobactam, sulfones sulbactam and penicillanic acid were also introduced. These inhibitors can be given together with the antibiotic as conjugate. For instance, augmentin is a conjugate of amoxicillin and clavulanic acid (Weber *et al.*, 1984; Lewis, 2013; Drawz *et al.*, 2014).

To clarify the problem of β-lactamase-antibiotic resistance in gram-negative bacteria, it is needing to know the history of β-lactams beginning with Alexander Fleming. It emerged in 4 different waves (Table 1.3). In the first wave included a few variant narrow-spectrum penicillinases that emerged in association with the use of ampicillin to treat gram-negative bacterial infections. The spread of strains elaborating these enzymes, such as TEM-1 of *E. coli*, prompted the development of newer β-lactam classes (such as the aztreonam, cephalosporins, and carbapenems) that were resistant to hydrolysis. The second wave of resistance which is of clinical importance happened in the 1980s and involved the emergence of resistance to extended-spectrum cephalosporins. Mutations in gram-negative bacteria continued, resulting in the expression of high-level resistance to extended-spectrum cephalosporins. The third wave involved the occurrence and spread of the CTX-M family of β-lactamases, these enzymes are cephalosporinases. The fourth wave of β-lactamase–antibiotic resistance is the spread of carbapenemases. The carbapenems are being hydrolysed by a large number of β-lactamases produced by gram-negative bacteria (Rice, 2012).

The most common mechanism of resistance to beta-lactam antibiotics is the production of beta-lactamases, which destroy beta-lactam antibiotics by binding to the PBPs, thus inhibiting the synthesis of peptidoglycan on the cell wall of the bacteria. There are three major mechanisms of beta-lactam resistance; decreased access to the PBPs reduced PBP binding affinity, and annihilation of antibiotic by the expression of β- lactamase (Ambler, 1980).

Table 1.3 Timeline is showing the use of various classes of antibiotics and describing resistance to that class of antibiotic in formerly susceptible microbes (Rice, 2012).

1942	1949	1962	1966	1979	1985	1990	1993
First clinical use of penicillin.	Osteomyelitis due to penicillin-producing *Staphylococcus aureus.*	First clinical use of ampicillin.	Description of TEM penicillinase.	First clinical use of cefotaxime.	First clinical use of imipenem.	Description of CTX-M.	First carbapenemase from Enterobacteriaceae.

1.2.4.3.3 Classification of ESBL

In 1995, Bush et al. devised a classification of β-lactamases based on their functional characteristics and substrate profile (K. Bush *et al.*, 1995). This classification of the ESBL enzymes is divided into three groups. The first group is cephalosporinases, which are not inhibited by clavulanic acid. The second group is broad-spectrum enzymes, which are generally inhibited by clavulanic acid. The third group is metallo-β-lactamases (Keat, 2011). At present, β-lactamases have been classified by two classification schemes. β-lactamases have been grouped on the basis of antibiotic resistance profiles in the Bush-Jacoby-Medeiros scheme (Bush *et al.*, 1995; Bush and Jacoby, 2010) and hence it is of greater clinical significance than the Ambler scheme which classifies the β-lactamases on the molecular basis (Ambler, 1980; Hall and Barlow, 2005). On the basis of sequence homology, the Ambler classification scheme categorises β-lactamases into four groups, i.e. A, B, C and D as presented in (Table 1.4) (Ambler,

1980; Ambler *et al.*, 1991; Hall and Barlow, 2005; Thirapanmethee, 2012). Different β-lactamases with active-site serine fall under the A, C and D group while group B is constituted by metallo-β-lactamases (MBLs) that demonstrate a unique evolutionary origin. MBLs contain one active zinc site at least (Hall and Barlow, 2005).

Another classification scheme was put forward in 2009. In this scheme, the description of ESBLs was expanded thereby permitting the inclusion of clinically significant acquired β-lactamases which are capable of acting against extended-spectrum cephalosporins and/or carbapenems. Considering the simplicity of this scheme which had the chances of missing minute disparities between β-lactamases, it was stated that the scheme was not put forward with the objective of replacing the available schemes. The primary aim of the scheme was to complement other schemes in the context of healthcare (Giske *et al.*, 2009). The Ambler scheme and the Bush-Jacoby-Medeiros scheme continued to be used in the meantime. More than 1000 β-lactamases have been discovered (Malloy and Campos, 2011; Bush *et al.*, 2015).

Table 1.4 Classification of β-lactamases according to Ambler molecular scheme (Thirapanmethee, 2012).

	Class	β-lactamases	Examples
Serine β-lactamases	A	ESBL TEM- type	TEM-1, TEM-2, and TEM-3
		ESBL SHV- type	SHV-1 and SHV-5
		ESBL CTX-M- type	CTX-M1 and CTX-M9
		Carbapenemases	KPC
	C	AmpC cephamycinases (chromosomal encode)	AmpC
		AmpC cephamycinases (plasmid encode)	CMY and DHA
	D	Broad spectrum β-lactamases	OXA-1 and OXA-9
		ESBL OXA- type	OXA-2 and OXA-10
		Carbapenemases	OXA-48 and OXA-23
Metallo β-lactamases	B	Metallo β-lactamases	VIM and IMP

1.2.4.3.3.1 TEM

The largest group of ESBL enzymes is the TEM family (Bradford, 2001). According to Ambler scheme classification, which is based on the nucleotide and amino acid sequences in TEM enzymes, TEM belongs to class A. TEM-1 was first reported in 1965 from an *E. coli* isolate from a patient in Greece (Datta and Kontomichalou, 1965). TEM-1 is the most common β-lactam inactivating enzyme found in enteric bacilli, especially in *E. coli* and *K. pneumoniae*, and is also seen with increasing frequency in other gram-negative species (Bradford, 2001; Hujer *et al.*, 2009). TEM is inhibited by clavulanic acid and can hydrolyse ampicillin more than carbenicillin, oxacillin or cephalothin (Paterson and Bonomo, 2005). When ceftazidime first became available for clinical use in 1982, only TEM-1, TEM-2 and SHV-1 were known. Now, over 170 modified derivatives of TEM-1 and TEM-2 are being circulated and demonstrate

resistance to monobactams and 2nd and 3rd generation cephalosporins. They may also resist the activity of β-lactamase inhibitors (Bush and Jacoby, 2010; Salverda *et al.*, 2010).

1.2.4.3.3.2 AmpC

AmpC β-lactamase is clinically significant though it is not an ESBL (Jacoby, 2009). It has been suggested that this enzyme is an archetypal β-lactamase on a structural basis (Burman *et al.*, 1973; Jaurin and Grundström, 1981). Though it is structurally different from penicillinases and TEM, its active site also contains serine (Jaurin and Grundström, 1981). Chromosomes of Enterobacteriaceae and some other bacteria contain genes for these enzymes (Jacoby, 2009). Still, plasmid-borne AmpC genes like CMY-8, CMY-9 and CMY-19 have also been reported, and these must have contributed in the spread of these enzymes to other bacteria with a poor expression of the *blaAmpC* gene.

AmpC-type β-lactamases are under molecular class C in Ambler's classification scheme. AmpC β-lactamases mediate resistance to most penicillin antibiotics, as well as extended-spectrum cephalosporins and β-lactamase inhibitor-β-lactam combinations (Jacoby, 2009). Cefoxitin resistance is used as an indicator of AmpC-mediated resistance, but it can also be an indication of loss of outer membrane permeability (Philippon *et al.*, 2002). AmpC is usually encoded on the chromosome of gram-negative bacteria, including *Citrobacter freundii*, *Enterobacter cloacae* and *Morganella morganii*. The chromosomal AmpC gene expression in these organisms is inducible by β-lactam antibiotics, such as cefoxitin and imipenem, however poorly induced by third- or fourth-generation cephalosporins. In *E. coli* and *Shigella spp*., AmpC is usually constitutive, minimal and poorly expressed, whereas in *Klebsiella* and *Salmonella spp*., the *AmpC* gene is missing from the chromosome (Schmidtke and Hanson, 2006; Jacoby, 2009). AmpC enzymes have been named according to the resistance produced to antibiotics, such as the resistance provided to cephamycins (CMY), cefoxitin (FOX) and moxalactam (MOX); according

to the site of discovery, such as the Dhahran Hospital in Saudi Arabia (DHA); and according to the patient name Bilal (BIL) (Philippon *et al.*, 2002).

1.2.4.3.3.3 OXA-48

OXA β-lactamases belong to class D according to Ambler classification (Ambler *et al.*, 1991). The OXA group usually occurs in *Acinetobacter* and *Pseudomonas spp.* OXA β-lactamases attack oxyimino-cephalosporins and have a higher hydrolytic activity against oxacillin, methicillin and cloxacillin more than against benzylpenicillin (Rasmussen and Høiby, 2006). Presently, over 180 different variants of OXA enzymes have been identified based on the protein level (http://www.lahey.org/studies/). Most OXA-types β-lactamases are not regarded as ESBLs because OXA does not hydrolyse extended-spectrum cephalosporins. OXA-11 is the first extended spectrum of *P. aeruginosa* isolated in October 1991 from the blood cultures of a burns patient in Turkey (Hall *et al.*, 1993). OXA-10, as well as its derivatives, have been found around the world (Al-Agamy *et al.*, 2012; Vatcheva-Dobrevska *et al.*, 2013; Farshadzadeh *et al.*, 2014; Hansen *et al.*, 2014). Most OXA-type ESBLs derive from OXA-10 (OXA-11, OXA-13, OXA-14, OXA-16, OXA-17 OXA-19 and OXA-28); from OXA-2 (OXA-15 and OXA-32) to a lesser extent; or others unrelated to any recognised broad-spectrum OXA enzymes (OXA-18 and OXA-45) (Naas *et al.*, 2008). Rasmussen and Hoiby sub-classified carbapenem-hydrolysing OXA enzymes into eight distinct branches or subgroups, namely, OXA-23, OXA-24, OXA-48, OXA-50, OXA-51, OXA-55, OXA-58 and OXA-60 (Walther-Rasmussen and Høiby, 2006).

An increment in resistance to carbapenem involves the role played by OXA-derivatives (Bush and Jacoby, 2010; Evans and Amyes, 2014). A number of bacterial species all across the globe have been found to contain the OXA carbapenemases which have been categorized into sub-divisions namely OXA-23, OXA-24/40, OXA-48, OXA-51 and OXA-58 (Carrer *et al.*, 2010; Castanheira *et al.*, 2011; Kalpoe *et al.*, 2011; Evans and Amyes, 2014; Smith *et al.*, 2014;

Alyamani *et al.*, 2015; Cakirlar *et al.*, 2015; Kamolvit *et al.*, 2015; Shrestha *et al.*, 2015; Tsiatsiou *et al.*, 2015).

Horizontal Gene Transfer (HGT) always occurs between *E. coli* strains and is the most common cause of increasing virulence potential and antimicrobial resistance (Juhas, 2015). The crystal structure of OXA-48 has been demonstrated, showing how this enzyme has a different mechanism for carbapenem hydrolysis. OXA-48 does not possess a hydrophobic bridge across the active site. The active-site cleft of OXA-48 has a larger hydrophobic region that allows the hydroxyethyl group of meropenem greater mobility (Evans and Amyes, 2014).

1.2.4.3.4 Epidemiology of ESBL positive bacteria

The CTX-M ESBL group has turned into the most common ESBL type in the family of Enterobacteriaceae in community-acquired as well as nosocomial infections since the early 2000s. Previously, ESBL variants of TEM and SHV held this position (Cantón *et al.*, 2012). Still, different regions of the world have different predominant ESBL types. Reports have indicated transmission of ESBL-carrying infections in healthcare centres and community (Burke *et al.*, 2012; Baraniak *et al.*, 2013; Valenza *et al.*, 2014).

Western Europe was the first to apply the utilisation of extended-spectrum β-lactam antibiotics clinically; this was followed by rapid increment and spreading of ESBL strains in Europe and then in the United States and Asia (Bradford, 2001). The incidence of ESBL infections was found to be lesser in European healthcare centres in comparison to other regions. This incidence of ESBL infections has been reported by a study conducted in 2007 that analysed nosocomial infections in 266 healthcare centres in Europe, Latin America, North America and Asia (Reinert *et al.*, 2007). The highest prevalence of ESBL in Enterobacteriaceae was reported by Greece and Poland. Since that time, clinical isolates with ESBLs have been reported in many

countries which seemed to be free from ESBL enzymes Germany (Valenza *et al.*, 2014), France (Ruppé *et al.*, 2012; Arnaud *et al.*, 2015), the United Kingdom (Wickramasinghe *et al.*, 2012; Horner *et al.*, 2014), Ireland (Burke *et al.*, 2012), Finland (Nyberg *et al.*, 2007), Norway (NASEER *et al.*, 2009), Sweden (Brolund *et al.*, 2014), Denmark (Hansen *et al.*, 2012) and Austria (Huemer *et al.*, 2011).

Prevalence of antibiotic resistance and particular those mediated by ESBL has increased in Saudi Arabia and rest of the Middle East just like other regions of the globe (Jean and Hsueh, 2011; Zowawi *et al.*, 2013). Researchers in Saudi Arabia have been exploring ESBL-mediated antibiotic resistance in different settings in the community as well as healthcare centres (Zowawi *et al.*, 2013). Besides the detection of common genes like OXA-48 in community-acquired as well as nosocomial isolates (Carrer *et al.*, 2010; Jean and Hsueh, 2011; Newire *et al.*, 2013; Zowawi *et al.*, 2013; Zamani *et al.*, 2015). In general, ESBLs have been found in clinical isolates of Enterobacteriaceae (Al-Agamy *et al.*, 2012; Zowawi *et al.*, 2013; Somily *et al.*, 2014; Alyamani *et al.*, 2015). Al-Agamy et al. researched two hospitals of Riyadh (The capital of Kingdom of Saudi Arabia) involving analysis of 400 samples of *K. pneumoniae* and found that prevalence of ESBL strains was 55% and SHV enzymes were predominant (Al-Agamy *et al.*, 2009). Conversely, another study by Tawfiq checked 430 isolates of *K. pneumoniae* in Al-Qassim and reported a lesser prevalence of ESBLs (Tawfik *et al.*, 2011). In particular, 89.1% isolates contained genes for SHV, and most of them contained SHV-12. 70.9% isolates possessed the TEM genes. Al-Agamy et al. checked 152 isolates of *E. coli* and prevalence of ESBL was found to be 20.39%. Out of 31 isolates, 30 possessed CTX-M-15 (Al-Agamy *et al.*, 2014). A distressing trend noticed in Saudi Arabia is the evolution of new β-lactamase genes which are responsible for carbapenems resistance in species other than *P. aeruginosa*. blaOXA-51, for instance, OXA-48 has been detected in *K. pneumonia* isolates in Riyadh. These isolates demonstrated lesser sensitivity to carbapenem. OXA-23-like gene has been detected in Eastern

part of Saudi Arabia in *Acinetobacter calcoaceticus-baumannii* complex strains which are resistant to carbapenem (Shibl *et al.*, 2013; Alyamani *et al.*, 2015; El-Mahdy *et al.*, 2017).

Oman, UAE and Kuwait have also witnessed elevation in resistance to carbapenem in Enterobacteriaceae strains. Majority of the OXA-48 has been isolated from specimens collected from individuals who had not travelled to other regions of the world which implies that autochthonous infections are predominant. Moreover, these states can serve as reservoirs of these strains, and from these regions, the strains demonstrating resistance to carbapenems can spread to the rest of the world (Sonnevend *et al.*, 2015). Considering these trends, it is most necessary that efforts are made for better surveillance of Enterobacteriaceae strains which are resistant to carbapenem in the Middle East and the Arabian Peninsula (Jamal *et al.*, 2016).

In a nutshell, Saudi Arabia, as well as other parts of the Middle East, is witnessing serious developments in antibiotic resistance such as increment in the prevalence of CTX-M and OXA-48 ESBL genes. In particular, the CTX-M-15 and OXA-48 genes found in Enterobacteriaceae, mostly in *E. coli*, are getting more prevalent in community-acquired as well as nosocomial infections.

1.2.4.3.5 Treatment

Treating infections caused by ESBL producing bacteria has proved to be a challenge for clinicians. Usually used antibiotic classes, such as penicillins, third-generation cephalosporins and monobactams, are often rendered ineffective for these types of infections. A study by Auer et al. explained this point (Auer *et al.*, 2010). The oral therapy is given to community patients having urinary tract infections caused by ESBL producing *E. coli*. The resistance rates to ciprofloxacin and trimethoprim-sulfamethoxazole were above 70% while those for gentamicin were around 21%. Also, in this study, these included fosfomycin, for which the bacteria

exhibited 97% susceptibility, nitrofurantoin, 94% susceptibility and ertapenem, 100% susceptibility (Auer *et al.*, 2010). Pivmecillinam is an oral antibiotic that has been used extensively within Nordic countries. Evidence suggests that pivmecillinam has activity against ESBL producing organisms (Dewar *et al.*, 2014). Carbapenems, for example, meropenem and imipenem, are the treatment of choice for patients suffering from ESBL related urosepsis and severe sepsis (Pallett and Hand, 2010).

Increased reports of carbapenemase activity have also been noted in areas such as London, an outbreak between 2008 and 2010 described 20 *K. pneumoniae* isolates carrying the OXA-48 determinant in 13 patients (Thomas *et al.*, 2013). OXA-48 resistance has increased from one confirmed case in 2007 to 179 confirmed cases in 2013 (Public Health England, 2016a). Many studies were initiated to determine relationships between antibiotic overuse and the potential to forecast future outbreaks.

1.2.5 Bacterial interaction and invasion of cells

1.2.5.1 Normal Human Urothelial Cell Line (TRET-NHUC)

Normal human urothelial cell line (TRET-NHUC) was kindly provided by Prof. Knowles, from Cancer Research UK Clinical Centre, St. James's University Hospital in Leeds. Normal human urothelial cells (NHUC) are cells covering the mucosal layer of the urinary tract (Hickling *et al.*, 2015). At the molecular level, NHUC covering the ureters, bladder and the urethra are morphologically similar but have different embryological derivations (Al-Kurdi, 2017). Data from recent studies indicate some degree of difference with regards to the morphological and biochemical features of NHUC lining different parts of the urinary tract (Hickling *et al.*, 2015).

1.2.5.2 Bacterial interaction with cells

Usually, the urinary tract is a sterile environment, using different host mechanisms to prevent bacterial colonisation and survival. The most pathogenic bacteria that cause UTIs are flora from the bowel, which enters the bladder by the urethra. UTI strains have a repertoire of adhesins that enable the bacteria to adhere to the cellular surfaces (Bien *et al.*, 2012). Therefore, the first line of host defence against UTIs is focused on preventing bacterial adherence to the bladder mucosa.

At first, the bacteria make a reversible contact which later becomes irreversible through physical and chemical changes. Yet, the contact is formed only when adhering bacteria reach near the surface. Bacteria may reach the surface through different physical forces such as hydrophobic interactions, van der Waal's forces and Brownian motion. They may also have utilised certain other mechanisms like sedimentation, active and convective transport utilising turbulent flow and flagella (Gottenbos *et al.*, 2002; Palmer *et al.*, 2007). Many factors affecting these interactions include the surrounding solution, the surface of the substrate and surface of bacterial cells (Parent and Velegol, 2004). Also, adhesion of bacteria is dependent on several other factors from host, bacteria and environment.

The gram-negative bacterium which is mostly isolated from UTI patients is the UPEC. Motility proves to be a crucial factor for interacting the bacteria to the cells. Using its flagella, UPEC is capable of swimming in the fluid as well as semi-fluid media. Type 1 fimbriae, P fimbriae and S fimbriae are the surface virulence factors that facilitate adherence of bacteria and hence are involved in bringing about UTI. Virulence of UPEC also involves the role of lipoprotein α-HlyA. Hlya is common in gram-negative uropathogens. Also, Hlya is a toxin which is discharged by bacteria and transmitted to the site of activity (Bien *et al.*, 2012).

1.2.5.3 Invasion and colonisation

The urinary bladder is formed of mucosal, submucosal, muscular and a perivesical soft tissue layer. The mucosal layer is known as NHUC. Usually, the bladder urothelium renews itself every 3 to 6 months, however during bacterial infection damage; the urothelium regenerates completely within 72 hours (Ho *et al.*, 2012). Attachment and invasion happen by specific interactions between UPEC's FimH adhesion and urothelial cell proteins (UPs) (Thumbikat *et al.*, 2009). UPs are important integral membrane proteins. They are mediators in UPEC cell invasion and also used as biomarkers for differential diagnosis of urothelial cancer (Wu *et al.*, 2009).

Many studies (Justice *et al.*, 2004; Garofalo *et al.*, 2007; Rosen *et al.*, 2007; Thumbikat *et al.*, 2009; Robino *et al.*, 2013) have been conducted to explore the mechanism of action of UPEC on bladder epithelial cells. In a mouse model of UTI, it has been proved that UPEC interacts with urothelial cells by a multi-step pathogenic pathway to establish bacterial colonisation. UPEC uses type 1 pili as a receptor to adhere to the bladder mucosa. After attachment, the bacteria are able to invade and replicate within the cytoplasm of the facet cells. Within these cells, UPEC forms intracellular bacterial communities (IBCs), which that help adhere to other cells and promote establishment and recurrence of UTI (Rosen *et al.*, 2007). In a study, Scott and others have characterised three phases of IBC formation in a mouse model. The first phase consists of attachment and invasion of uropathogens into NHUC. In the second phase, IBCs mature and form closely packed tight junctions. The last phase is characterised by fluxing and release of uropathogens (Scott *et al.*, 2015). Moreover, uropathogens can form reservoirs in the layers of NHUC. In addition to pathogenic virulence factors that promote colonisation, various other predisposing factors assist pathogenic adherence. These include alterations to the host's immune system like as seen in immunocompromised patients, anatomical and physiological factors, and antimicrobial use (Behzadi *et al.*, 2010).

1.2.5.4 Programmed Cell Death (PCD)

Intracellular bacteria have the ability to induce host cell cytotoxicity by secreting different types of virulence factors and by repressing host survival pathways (Lu *et al.*, 2013). Cellular cytotoxicity can lead to cell death by apoptosis or necrosis. Both apoptosis and necrosis are a programmed form of cell death; however, there are significant differences between them. Apoptosis is a programmed form of cell death which is required for the growth and the survival of multicellular organisms. It can happen under both physiological and pathological conditions. On the other hand, necrosis is a pathway of cell death related to cell injury and is always pathological (Kumar *et al.*, 2015). When comparing with the necrosis, apoptosis does not enhance inflammatory responses which are induced with the necrosis process, such as, loss of cell membrane integrity and an uncontrolled release of products of cell death into the extracellular space (Rathmell and Thompson, 2002). Also, in the apoptotic cells, there are programmed effects on the cellular content, such as mitochondria, lysosomes and Golgi bodies. In contrast, the necrotic cell's membrane is lysed and ruptured; therefore, all the cell contents are released to cause inflammatory responses (Negroni *et al.*, 2015). It is well known that apoptosis and necrosis can coexist in the same cell type (Lu *et al.*, 2013). Early apoptotic cells are characterised by an intact cell membrane with cell surface expression of phosphatidylserine (PS) to aid recognition by phagocytes. When the membrane becomes permeabilised, early apoptotic cells become late apoptotic cells, which can then progress on to necrotic cells (Poon *et al.*, 2010).

There are morphological differences between apoptosis and necrosis. Apoptosis is characterised by shrinkage of cells, nuclear changes such as nuclear fragmentation and no adjacent inflammation. However, swelling of cells, chromatin condensation, disrupted plasma membrane, leakage of cellular contents is characteristic of necrosis (Kumar *et al.*, 2015). These morphological markers are useful in determining the stages of apoptotic cell death.

Depending on the type of virulence factor, cell death can happen via different mechanisms. In apoptosis, cell death can occur by signalling cascade pathways. One pathway is caused by the imbalance of B-cell lymphoma-2 (Bcl-2) family proteins causing increased mitochondrial permeability, leakage and caspase activation whereas the extrinsic or death receptor pathway is caused by the engagement of plasma membrane death receptors on cells leading to the formation of death-including signalling complex and lastly caspase activation. On the other hand, in necrosis, the mechanism of cell death is associated with its specific pathology. Phagocytosis and significant immune response are also characteristic of necrosis (Vanlangenakker *et al.*, 2008; Kumar *et al.*, 2015).

UPEC is responsible for most of UTIs. UPEC is known to affect apoptosis by the secretion of haemolysins (Lu *et al.*, 2013), especially a pore-forming toxin known as HlyA (Nagamatsu *et al.*, 2015). HlyA forms a pore in cell membranes causing urothelial cell toxicity and finally cell lysis.

In 2009, Santos and his colleagues concluded that *Acanthamoeba*, free-living amoebae were found in 26% of urine samples under investigation. Previous studies showed that the pathogenic *E. coli* K1 interact with *Acanthamoeba* and have the ability to invade and remain viable inside *Acanthamoeba* (Alsam *et al.*, 2006). *Acanthamoeba* resembles macrophages in many ways like the cell surface receptors. From the previous, that suggests *Acanthamoeba* may offer a good model for studying *E. coli* pathogenesis and learning about its immune evasion mechanisms (Cardas *et al.*, 2012).

1.3 *Acanthamoeba*

1.3.1 Background

Acanthamoeba is an opportunistic microscopic single-celled living organism, which was isolated before 1930. However; a definitive description was presented by Castellani in 1930 (Siddiqui and Khan, 2012). Also, in 1930 Douglas named the amoeba isolated by Castellani, *Hartmanella castellanii*, and in 1931 Volkonsky returned it to the genus *Acanthamoeba* (Nero *et al.*, 1964). *Acanthamoeba* has unfolded everywhere the planet, and is isolated from soil, air, natural and treated water, from dental treatment units, hospitals and dialysis units, eyewash stations (De Jonckheere, 1991; Karamati *et al.*, 2016). There are two stages in the life cycle of *Acanthamoeba* were trophozoites or cysts. There are many species of *Acanthamoeba* including genotypes T1, T2, T3, T4, T5,T6, T10, T11, T12 and T15 can cause severe infections such as keratitis and encephalitis. They feed on bacteria and act as a Trojan horse for them (Santos *et al.*, 2009; Siddiqui and Khan, 2012; Guimaraes *et al.*, 2016). However, *Acanthamoeba* feeds on yeasts, algae and fungi.

1.3.2 Ecology

Acanthamoeba strains have spread all over the world, and have been isolated from air, soil, and water (De Jonckheere, 1991; Kreuzer *et al.*, 2006; Reyes-Batlle *et al.*, 2014; Todd *et al.*, 2015; Karamati *et al.*, 2016). They have been isolated from natural and treated water, seawater, swimming pools, sewage, bottled water, and drinking water (Jeong and Yu, 2005; Lorenzo-Morales *et al.*, 2006; Visvesvara *et al.*, 2007; Siddiqui and Khan, 2012; Al-Herrawy *et al.*, 2014; Maschio *et al.*, 2015; Sente *et al.*, 2016). Additionally, *Acanthamoeba* have been isolated from contact lenses (Martin-Navarro *et al.*, 2008; Ibrahim *et al.*, 2009; Gomes *et al.*, 2016), also isolation from the healthcare environment includes dental treatment units, hospitals, dialysis units, and eyewash stations (Dendana *et al.*, 2008; Niyyati *et al.*, 2009; Siddiqui and Khan, 2012;

Retana-Moreira *et al.*, 2015; Fukumoto *et al.*, 2016; Castro-Artavia *et al.*, 2017). Moreover, they have been isolated from dust, yeast, and air conditioning systems (De Jonckheere, 1991; Astorga *et al.*, 2011; Özpinar *et al.*, 2017). *Acanthamoeba* has been isolated from mammalian cell cultures, and from humans (Marciano-Cabral and Cabral, 2003).

1.3.3 Life Cycle

There are two stages in the life cycle of *Acanthamoeba*, the first stage is vegetative trophozoite stage with a diameter of 13-23μm, and the second stage is the dormant cyst stage of 5-20μm (Figure 1.6). During the trophozoite stage, *Acanthamoeba* feed on microbes, and the optimal conditions include the food supply, neutral pH (6-8), temperature around ~30°C, and 50-80mOsmol. Harsh conditions for *Acanthamoeba* result in cellular differentiation into a double-walled cyst form. *Acanthamoeba* has shown a cyst wall contains 33% protein, 4 - 6% lipid, 35% carbohydrates, and 20% unidentified materials (Dudley *et al.*, 2008; Khan, 2009) In trophozoite stage they have spine-like structures on their surfaces called acanthopodia, hence the name *Acanthamoeba*. The acanthopodia are formed from the cytoplasm (Bowers and Korn, 1968). In addition, the primary function of the acanthopodia is their importance in adhesion to surfaces and cellular movement (Siddiqui and Khan, 2012).

The trophozoites have a single nucleus with a nucleolus, cytoplasm with multiple elongated mitochondria, lysosomes, endoplasmic reticulum, ribosomes, Golgi apparatus and vacuoles. The inner wall consists of cellulose, while the outer wall possesses proteins and polysaccharides (Neff and Neff, 1969). The method of reproduction in trophozoites is by binary fission. On the other hand, under unfavourable conditions, such as lack of nutrients, overcrowding of cells in culture, high temperature and different pH and osmolarity, the trophozoite transform into inactive cysts. The cysts are double walled, composed of fibrous ectocysts and fine fibrils in a granular matrix, and called endocysts (Dudley *et al.*, 2008). Under favourable conditions, the cysts stage change to trophozoites to complete their life cycle (Lloyd, 2014).

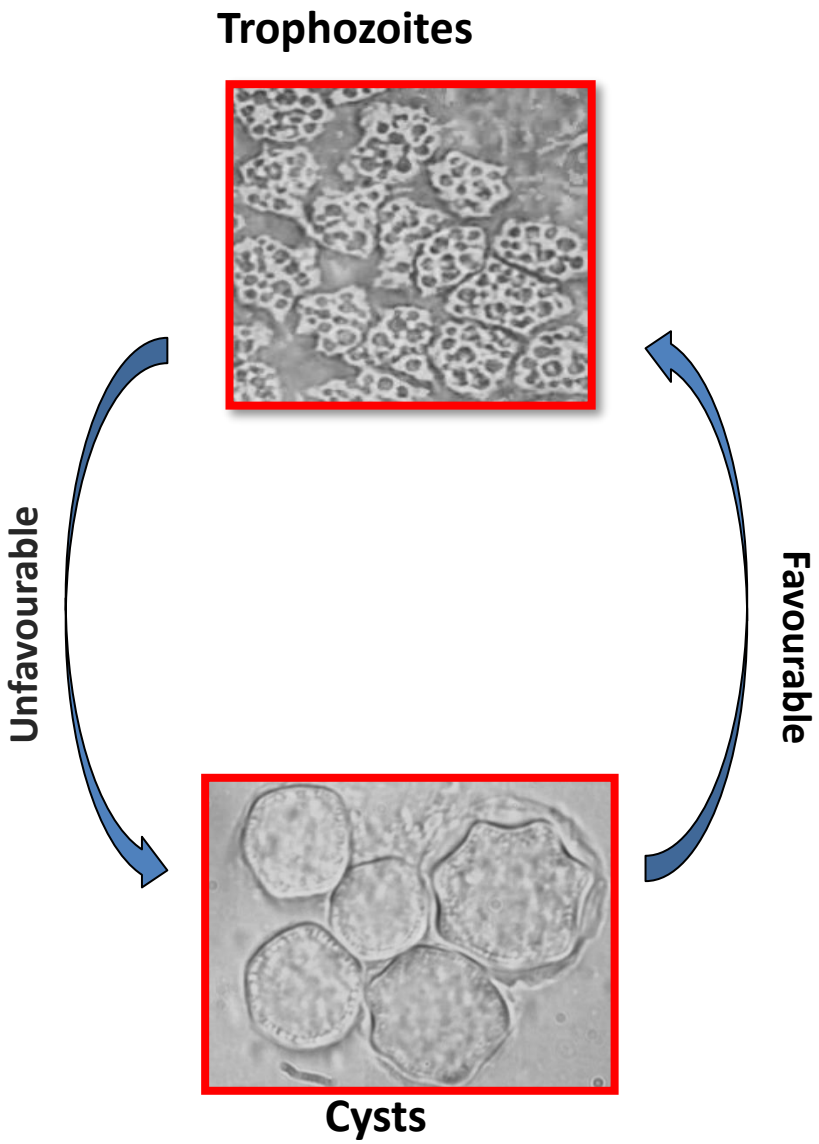

Figure 1.6 Micrographs showing the life cycle stages of *Acanthamoeba spp.* Bar = 10µm (Cardas *et al.*, manuscript in preparation).

1.3.4 Feeding

The main food of *Acanthamoeba* is bacteria, but it also feeds on yeasts, algae and fungi. They may use their acanthopodia to capture the food particles (Alsam *et al.*, 2006). Digestion happens in the food vacuoles and phagolysosomes (Abd *et al.*, 2009). A*canthamoeba* actively feeds in their trophozoite, and there are two pathways to uptake food: the nonspecific pathway of pinocytosis, which takes up soluble material, and specific receptors as in phagocytosis (Bowers

and Olszewski, 1972; Khan, 2009). They can uptake solutes of varying molecular weights such as albumin (Alsam *et al.*, 2005). It is known that *Acanthamoeba* preferentially feeds on gram-negative bacteria (Rosenberg *et al.*, 2009). Amongst gram-negative bacteria, *E. coli*, serves as an excellent food source (Huws *et al.*, 2005; Alsam *et al.*, 2006).

1.3.5 Classification of *Acanthamoeba*

Based on 18S rRNA gene sequences, the genus *Acanthamoeba* has been divided into 22 different genotypes (T1 – T22) (Adamska, 2016; Behera *et al.*, 2016; Taher *et al.*, 2018). Genotypes have 5% or more sequence divergence. Also, some researchers are trying to classify *Acanthamoeba* based on its small mitochondrial subunit ribosomal RNA (16S rRNA) gene sequences (Ledee *et al.*, 2003; Rahman *et al.*, 2013). The most common human infections due to *Acanthamoeba* have been associated with the T4 genotype. For example, more than 90% of *Acanthamoeba* keratitis (AK) cases due to T4 genotype (Arnalich-Montiel *et al.*, 2014). In addition, T4 has been associated with non-keratitis infections such as cutaneous infections (Siddiqui and Khan, 2012).

1.3.6 *Acanthamoeba* human infections

Acanthamoeba can cause severe infections, such as keratitis that can result in blindness, and *Acanthamoeba* Granulomatous Encephalitis (AGE), which involves the central nervous system (Alsam *et al.*, 2003; Sciences, 2008; Siddiqui and Khan, 2012; Padzik *et al.*, 2018).

1.3.6.1 *Acanthamoeba* keratitis (AK)

Acanthamoeba keratitis is associated with the production of tears, inflammation with redness, photophobia, stromal infiltration, and stromal opacity together with excruciating pain due to radial neuritis, epithelial loss and stromal abscess formation. Without proper treatment, it may lead to corneal ulcers or even blindness. As shown in pictures below (Figure 1.7), the patient who suffered from AK, lost 80% of his vision in one month of infection (Alsam et al., 2008).

The treatment of AK is problematic because of its recurrence. Until now, no chemotherapeutic has been described as a single effective treatment against AK, regardless of the isolate or genotype that causes it. This problem is because there are different factors, including the many virulence traits that different strains possess, which makes it almost impossible to establish a correlation between *in vitro* and *in vivo* efficacies. Treatment usually includes a biguanide in combination with a diamidine. This disease occurs most often in contact lens with wearers who do not disinfect their lenses. However, in some cases, *Acanthamoeba* has been reported in patients who are not lens wearers (Yousuf *et al.*, 2013; Lorenzo-Morales *et al.*, 2015; Omaña-Molina *et al.*, 2018; Padzik *et al.*, 2018).

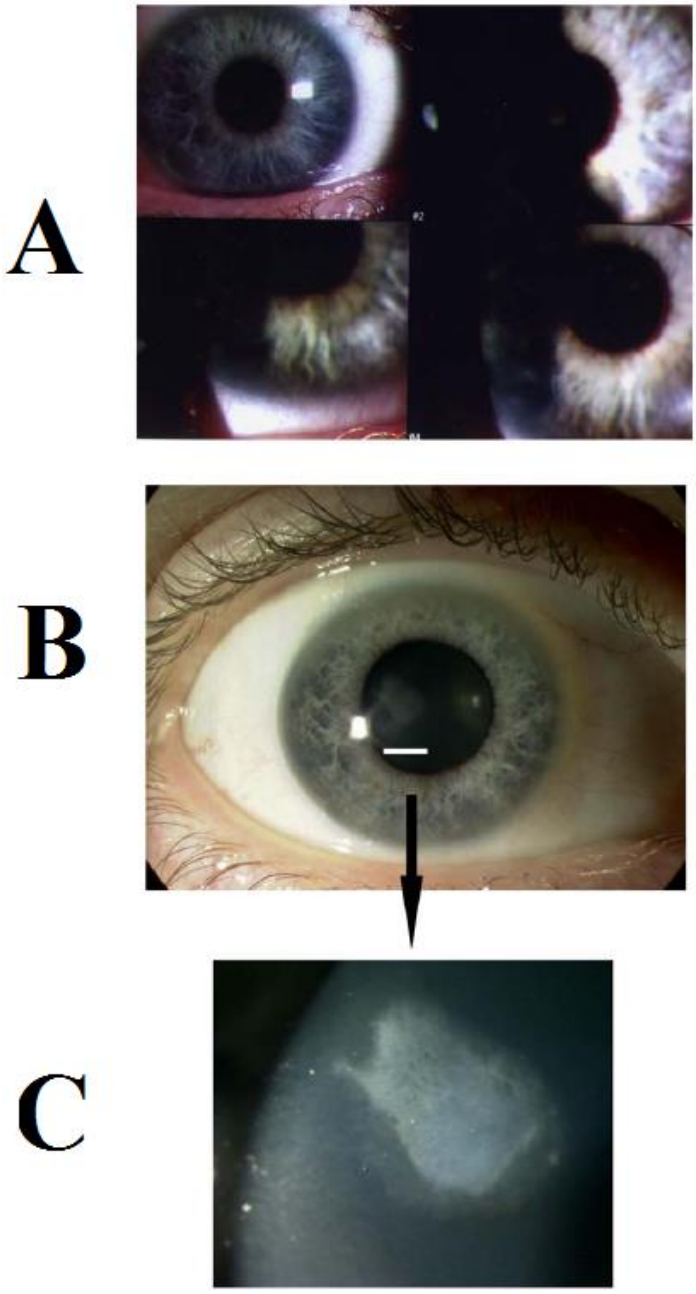

Figure 1.7 Cornea infected by *Acanthamoeba* keratitis.

(A) A picture of early epithelial infection with *Acanthamoeba* keratitis. (B) A picture for improvement of *Acanthamoeba* keratitis after six months of treatment with polyhexamethylene biguanide, propamidine- isethionate and neomycin. (C) A picture is displaying the affected area of the eye (cornea) (Alsam et al., 2008).

1.3.6.2 *Acanthamoeba* Granulomatous Encephalitis (AGE)

Acanthamoeba Granulomatous Encephalitis is a rare occurrence resulting in a fatal infection. The clinical features of this disease include stiff neck, headache, fever, nausea, hemiparesis, lethargy, vomiting, cranial nerve palsies, and then, death. Susceptible hosts include immunocompromised patients, as well as those with excessive use of steroids, diabetes, pneumonitis, renal failure, and liver cirrhosis. The pathology of autopsied brains shows severe oedema and haemorrhagic necrosis with this disease (Alsam *et al.*, 2003; Siddiqui and Khan, 2012; Coven *et al.*, 2017; Rodríguez-Pérez *et al.*, 2017; Matsui *et al.*, 2018; Sütçü *et al.*, 2018). It is difficult to diagnose AGE, because of the similarities of the symptoms with bacterial meningitis and viral encephalitis, however, scans by Magnetic Resonance Imaging (MRI) (Figure 1.8) (Matsui *et al.*, 2018). There are several drugs to treat AGE, such as ketoconazole, amphotericin-B, rifampicin, fluconazole, sulfadiazine, and trimethoprim-sulfamethoxazole (Kaushal *et al.*, 2008; Zamora *et al.*, 2014).

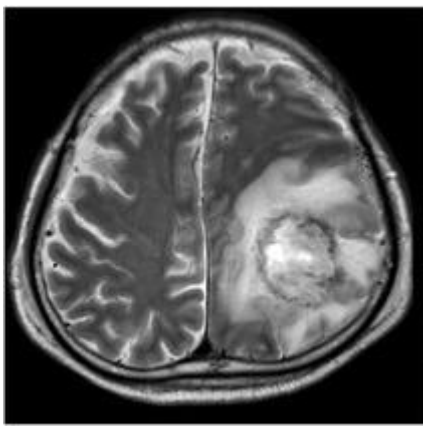

Figure 1.8 Brain imaging by MRI for a patient with granulomatous amoebic encephalitis caused by *Acanthamoeba* (Matsui *et al.*, 2018). Brain imaging demonstrates changes of cerebritis visible as T2 hyperintensity with the mass effect that involves the anterior temporal lobe progressing to affect most of the multiple areas of necrosis.

1.3.7 *Acanthamoeba* pathogenesis

The *Acanthamoeba* killing starts by adhesion to the host cells, followed by phagocytosis through the secretion of extracellular proteases and the killing of the host cell. The pathogenicity of *Acanthamoeba* infections is a complex process that includes greater adhesion properties, secreted cytotoxic products, invasion mechanisms and survival mechanisms (Arnalich-Montiel *et al.*, 2014; Omaña-Molina *et al.*, 2017). The pathogenesis of *Acanthamoeba* can be divided by using two mechanisms: contact-dependent and contact-independent mechanisms (Alsam *et al.*, 2003).

1.3.7.1 Contact-dependent mechanisms

The essential step of infection is adhesion. Adhesion in *Acanthamoeba* to host cells is used during initial step when proceeding to the inside tissue (Sissons *et al.*, 2005; Alsam *et al.*, 2006; González-Robles *et al.*, 2017; Lee *et al.*, 2017). Mannose-Binding Protein (MBP) helps in adhesion of *Acanthamoeba* to the host. Phagocytosis is the next step; it leads to host cell damage (Alsam *et al.*, 2003; Imbertbouyer *et al.*, 2004; Yoo and Jung, 2012; Kang *et al.*, 2018).

The MBP is a 400kDa transmembrane protein consisting of multiple subunits in the range of 130kDa (Panjwani, 2010; Kang *et al.*, 2018), and has been identified as being a major virulence protein for *Acanthamoeba* infection especially in AK (Garate *et al.*, 2005; Kang *et al.*, 2018). In addition, many studies have shown that *Acanthamoeba* binding is inhibited by free mannose (Morton *et al.*, 1991; Alsam *et al.*, 2003). Also, this way is associated with the uptake of food, and it is still essential in pathogenesis (Alsam *et al.*, 2005).

1.3.7.2 Contact-independent mechanism

The most virulent of the contact independent factors of *Acanthamoeba* is extracellular proteases that lead to the death of the host cells (Garate *et al.*, 2004; Khan, 2009). The proteases are a group of enzymes which leads to breaking down the peptide bonds on fibrinogen, albumin, haemoglobin, chemokines and cytokines. There are various types of proteases present in *Acanthamoebae* such as serine proteases, Metallo-proteases, and cysteine proteases (Dudley *et al.*, 2008; Lorenzo-Morales *et al.*, 2013). One of the proteases recognised was a 107 kDa serine protease (Khan *et al.*, 2000). However, serine proteases of differing molecular weight: 12, 40, 42, 55, 70, 85, 97, 130, 133 and 230 kDa are the predominant type of proteases secreted by many of *Acanthamoeba* genotypes (Dudley *et al.*, 2008). Serine protease plays an important role in tissue invasion (Khan *et al.*, 2000) catabolism of host proteins and both stimulation and evasion of host responses (Sissons *et al.*, 2006). They degrade glycoproteins and induce damage to collagen (de Souza Carvalho *et al.*, 2011). Also, metalloproteases play a role in *Acanthamoeba* pathogenicity, special in human infections. It has been suggested that a metalloprotease of approximately 150kDa produced by *Acanthamoeba,* it is responsible for increasing blood-brain barrier permeability (Alsam *et al.*, 2005).

Although serine proteases are predominant, another type of contact-independent mechanism is the secretion of phospholipases (PLs). There are many studies suggested that PLs may be involved in cell membrane disruption and cell lysis because of their ability to hydrolyse ester bonds in glycerophospholipids (Lorenzo-Morales *et al.*, 2015). There are five main types of PLs known A1, A2, B, C, and D (Matin and Jung, 2011). One study by Tripathi has shown that PL A2 from *Acanthamoeba* trophozoites induces apoptosis in corneal epithelial cells (Tripathi *et al.*, 2013). Pathogenic strains of *Acanthamoeba* produce increased levels of protease activity and bring about higher levels of cytotoxicity than non–pathogenic strains.

1.3.8 Bacterial correlation with *Acanthamoeba*

Bacteria can develop different relationships with amoeba, and the nature of this relationship is determined by environmental conditions and virulence of the two organisms. According to Jeon, there can be three outcomes of the association between amoeba and bacteria, i.e. symbiosis, destruction of amoeba or destruction of bacteria (Jeon, 1995). Researchers have found that certain bacterial species demonstrate greater virulence when grown with amoeba in comparison to the same bacterial species living in the natural environment. Some bacterial species which may develop a relationship with amoeba include *E.coli* (Alsam *et al.*, 2006), *Campylobacter jejuni* (Bui *et al.*, 2012), *Vibrio cholerae* (Abd *et al.*, 2009), *Shigella sonnei* (Jeong *et al.*, 2007), *Legionella pneumophila* (Cirillo *et al.*, 1994), and *Mycobacterium avium* (Cirillo *et al.*, 1997).

1.3.8.1 Bacteria and *Acanthamoeba* interactions

Acanthamoeba is known to be bacterial predators, and they play a crucial role in controlling the number of bacteria in the soil (Rodríguez-Zaragoza, 1994). Soil-inhabiting *Acanthamoeba* generate enzymes which cause lysis of cell wall of various bacteria (Weekers *et al.*, 1995). Several factors influence the survival and growth of amoebae such as the time in which bacterial cell is engulfed, the bacterial potential for binding with trophozoite stages (Marciano-Cabral and Cabral, 2003) and feeding tropism of amoeba (Bottone *et al.*, 1994).

Environmental conditions and virulence of bacteria determine if *Acanthamoeba* can interact with bacteria. The pathogenic strain of *E. coli K1* has been found to remain alive and even multiply inside *Acanthamoeba* as mentioned earlier. On the contrary, non-pathogenic strain K12 of *E. coli* was destroyed by the amoeba. Conversely, when there was food in the environment, strain K1 caused lysis of amoeba and demonstrated exponential growth as

compared to strain K12 which demonstrated minimal growth in the same condition (Alsam *et al.*, 2006).

Acanthamoeba can offer habitat to numerous different bacteria which can enter, proliferate and leave amoeba (Hundt and Ruffolo, 2005). Some examples are *Listeria monocytogenes* which causes Listeriosis (Akya *et al.*, 2009) and *E. coli* 0157 (Barker *et al.*, 1999). Researchers have reported an increase in a number of bacteria when living inside *Acanthamoeba* cells in comparison to bacterial cultures grown in the absence of *Acanthamoeba*. *Acanthamoeba* may offer protection to bacterial cells against negative factors like antibiotics and chlorination (Declerck *et al.*, 2010).

1.3.8.2 *Acanthamoeba* used as a Trojan horse to carry pathogenic bacteria

An intriguing fact regarding these relationships is that bacteria are capable of utilising amoeba not only as a host but as a vehicle for transference from one host to another. In order to distinguish these relations, researchers use different terms. “Carrier” is the term used when bacteria get attached to the amoebic surface. Conversely, “Trojan horse” is the term used when bacteria reside inside *Acanthamoeba* (Khan, 2008; Mengue *et al.*, 2017). The presence of *Acanthamoeba* in critical patients may be helping pathogenic bacteria in urine, protecting them against antimicrobial drugs, disinfectants, and host immunity. Inside *Acanthamoeba*, the pathogenic multi-drug resistant bacteria survive and replicate (Cardas *et al.*, 2012). In harsh conditions, *Acanthamoeba* becomes cysts which act as a carrier for spreading bacteria. Therefore, *Acanthamoeba* may help the multidrug-resistant bacteria to re-infect the urinary tract. Even in harsh conditions notably, the presence of medication bacteria will multiply inside *Acanthamoeba* and burse them. It was discovered that the pathogenic bacteria become more virulent and more resistant to antibiotics if they infect an amoeba and then emerge from it (Moon *et al.*, 2011;

Guimaraes *et al.*, 2016). Re-recurrence of UTIs is more common, with 27% of patients suffering another infection within six months and 44% of patients experiencing another infection within one year. Between one-third and one-half of the recurrent infections are caused by the same strain that initiated the infection. Thus, the recurrent infections will help the bacteria to become more resistant to antimicrobials (Hilbert, 2013).

1.4 Current treatment

The utilisation of antibiotics in future is at risk owing to the ongoing increase in resistance to antibiotics, recurrent UTIs (rUTIs) and ESBL+ve strains. For that reason, treatment and prophylactic measures for UTIs that lack use of antibiotics has attracted researchers and clinicians (Beerepoot and Geerlings, 2016). Adherence and colonisation of bacteria are the processes which are mainly targeted by the latest preventive measures (Beerepoot and Geerlings, 2016).

Foxman and Buxton have successfully used *Lactobacillus* preparations for inhibiting the uropathogens to colonise the bowel. In this way, the risk of getting UTI through the ascending route is minimised. The efficiency of *Lactobacillus* preparations in reducing rUTIs has also been reported by clinical trials performed in the United States and the Netherlands (Foxman and Buxton, 2013). Similarly, Barber has reported quite fruitful results of using pilicides and mannosides in their *in vitro* experiments for targeting type 1 pili and adhesion protein FimH respectively leading to inhibition of bacterial adhesion (Barber *et al.*, 2013). Researchers have also found that when a postmenopausal female receives oestrogen replacement therapy, it enhances the number of Lactobacilli in the vagina which is accompanied by a reduction in vaginal colonisation by Enterobacteriaceae. Based on the previous discussion, that leads to a reduction in UTI incidence (Beerepoot and Geerlings, 2016). Moreover, adherence of *E. coli* to

the urothelial cells has been reported to be inhibited by fructose and a polymeric substance found in cranberries like Flavan-3-ols and Proanthocyanidins (Blumberg *et al.*, 2013). A number of research studies have reported the utilisation of several different medicinal herbs owing to their activities against bacteria (Lüthje and Brauner, 2016).

1.4.1 Alternative therapeutic techniques

A promising strategy which is still under research is incorporation of QACs into polymers for making antibacterial biomaterials (Abs) (Jiao *et al.*, 2017). In general, QACs represent a large group of cationic surfactants which are utilised as components of several industrial and household products (Zhang *et al.*, 2015). $N^{+}R1R2R3R4X^{-}$ is the formula which represents the structure of all QACs. In this formula, N represents the Nitrogen atom covalently linked with four distinct groups. Moreover, R can be a hydrogen atom, an alkyl group or a substituted alkyl group. Finally, X stands for an anion which is a halide in most cases. The majority of QACs are bromide or chloride salts while some are iodide salts as well (Jiao *et al.*, 2017).

The current literature also contains evidence for the antimalarial (Basilico *et al.*, 2015), and anticandidal (Xian *et al.*, 2016) activity of QACs. Besides their antibacterial activity against a broad range of bacteria, QACs can be structurally altered for different functions. That is the reason why they are increasingly used in various consumer products. For the same purpose, numerous generations of QACs have been formed (Melin *et al.*, 2014). Also, QACs was an effect on the growth of *Acanthamoeba* (Shirai *et al.*, 2000).

QACs mostly target the cytoplasmic membrane of the bacterial cell. Through their alkyl side chains, the QACs link to the cell membrane and damage it (Sütterlin *et al.*, 2008). All QACs are known to cause damage to cell membranes leading to leakage (Ioannou *et al.*, 2007). The

electron density of the ammonium nitrogen atom and the adsorbability and hydrophobicity of QACs are the factors that determine their AB activity (Kourai *et al.*, 2006). Ioannou et al., (2007) reported that the efficiency of QACs containing a hydrophobic chain of 16-carbon atoms in damaging the cell wall of gram-negative bacteria is greater than the efficiency of QACs with shorter chains.

1.4.2 Cetylpyridinium chloride (CPC)

Cetylpyridinium chloride (CPC) is a cationic quaternary ammonium salt of the pyridinium group used in different types of antiseptic mouthwash and lozenges (Savas *et al.*, 2015). Besides this, CPC is also employed as a disinfectant in public as well as private areas (Imai *et al.*, 2017). CPC has the molecular formula $C_{21}H_{38}NCl$ and a melting point of 77°C when anhydrous or 80–83°C in its monohydrate form, as shown in Figure (1.9) (Zarei *et al.*, 2013). It is an antimicrobial agent that kills bacteria and other microorganisms, and with 0.07% and 0.05% concentration has shown antibacterial activities (Chandrdas *et al.*, 2014). The effect of CPC happens by combining with negatively charged proteins on bacterial cells and breaking their cell membrane by disturbing the electrical balance (Savas *et al.*, 2015). Some bacteria, such as *Streptococcus spp*., *Staphylococcus aureus* and *E. coli* showed higher minimum inhibitory concentration (MICs) from 100µM to 2mM, these studies help CPC-containing drugs possibly useful for the treatment or the prevention of transmission of the pathogen (Bereswill *et al.*, 1999).

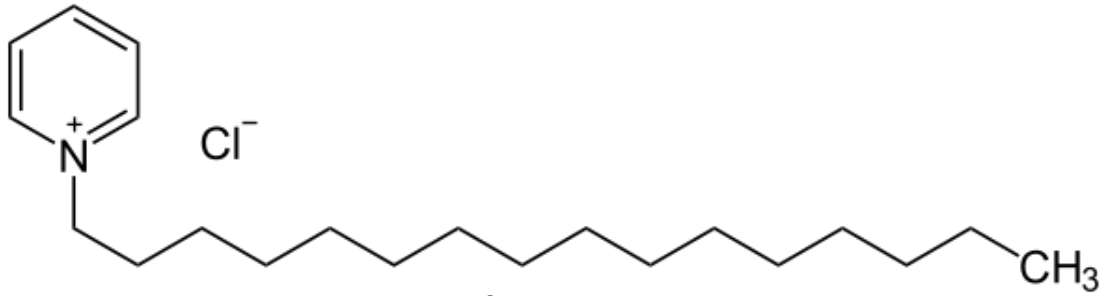

Figure 1.9 The chemical structure of cetylpyridinium chloride (CPC) (Zarei *et al.*, 2013).

The mode of action of CPC is dependent upon the capacity of this positively charged molecule to interact with negatively charged anionic sites on the cell walls of bacteria. Usually, bacterial cells carry a net negative charge. When bacteria are exposed to CPC, the positively charged molecule associates with the negative charge on the surface of the bacterial cell allowing the hydrophobic portion of CPC to interact with the cell membrane resulting in leakage of cellular components, inhibition of cell growth, and finally cell death (Cutter *et al.*, 2000; Mehall *et al.*, 2015).

In recent years, the antimicrobial activity of CPC has been studied in two main fields which are poultry processing (Arritt *et al.*, 2002; Waldroup *et al.*, 2010) and oral care (Hwang *et al.*, 2013). Based on findings from this project, it is believed that CPC can be studied for its utility in treating UTIs, specifically ESBL+ve strains.

1.5 Aims and objectives

ESBL positive and negative strains of *E. coli* are uropathogens which are commonly associated with UTIs including rUTIs. The main pathogenetic mechanisms are by intracellular bacterial in urothelial cells. *Acanthamoeba* is a Free-living amoeba (FLA) widely distributed in the environment and acts as a reservoir for a range of pathogens. It causes life-threatening human infections and has also been isolated from apparently healthy people. The role of *Acanthamoeba* in UTIs is a new topic and studying the interaction of ESBL+ve strains of *E. coli* with *Acanthamoeba* and urothelial cell line will shed some light on this field.

The development of increased resistance against available antibiotics is a major global concern and is a major threat to the successful treatment of infectious diseases. The indiscriminate use of various antimicrobial drugs for infectious disease treatment has resulted in the drug resistance in pathogens in recent years. Therefore, looking for new or additional methods and compounds for treatment is urgent. Also, using novel compounds CPC for therapy of both *Acanthamoeba* and ESBL+ve strains may help many patients suffering from prolonged and recurrent of UTIs. The results of the study will help create awareness about the problem of ESBLs production. Identification of specific proteins that contribute to pathogenicity by protein profiling in ESBLs producing bacteria can assist in the selection of appropriate novel compounds for the treatment of infection. So, this will lead to a reduction in length of hospital stay and reduce treatment costs for the patient.

The main issue explored in this thesis is the relationship between uropathogens (ESBL+ve strains) and pathogenic *Acanthamoeba* (T4), and to determine if this association may be one of cause and effect with regards to UTIs and rUTIs.

The major aims of this thesis were to

- Investigate the role of ESBL producing strains of *E. coli* in recurrent urinary tract infections.
- Check the presence of *Acanthamoeba* in ESBL+ve urine samples collected from patients diagnosed with UTI.
- Explore the interaction of ESBL producing strains of *E. co*li with *Acanthamoeba castellanii* (T4 genotype).
- Investigate the effect of bacterial and amoebic infections on the urothelial cell line.
- Explore the effect of cetylpyridinium chloride (CPC) on bacterial and amoebic infections on the urothelial cell line.

CHAPTER TWO 2

2. Characterisation of *E. coli* multi-drug resistant bacteria (TEM, AmpC, and OXA-48)

2.1 Introduction

It is well established in the medical community that Urinary Tract Infections (UTIs) are the most common bacterial infections seen in clinical practice in Europe and North America (Kucheria *et al.*, 2005). A study by Plowman et al. found that over 12 months, UTIs had the highest incidence (35%) of all nosocomial infections in a district general hospital in the United Kingdom, in patients over 60 years of age (Plowman *et al.*, 2001). Females are the most commonly affected group (around 50–60% of women will develop UTIs in their lifetimes (Al-Badr and Al-Shaikh, 2013), while other high-risk groups include children, the elderly, people with structural abnormalities of the urinary tract, and those having a urinary tract intervention such as catheter insertion (Ali *et al.*, 2009).

Many different factors can increase the incidence of UTIs, for example, frequent intercourse spermicide usage, injuries from trauma or surgery, as well as the insertion of urinary catheters (Nicolle, 2001, 2014; Morgan, 2007). The organisms that cause UTIs are found in many species of gram-negative Enterobacteriaceae and are often related to the lipopolysaccharide (LPS) layer of the gram-negative bacterial cell envelope (Madigan *et al.*, 2009). UTIs are caused by a variety of pathogens including *E. coli* (Flores-Mireles *et al.*, 2015). *E. coli* that cause UTIs are classified as uropathogenic *E. coli* (UPEC), and these strains account for 75–90% of uncomplicated UTIs, and can also cause bacteraemia, sepsis, and meningitis (Ahmed *et al.*, 2014).

Resistance to β-lactams is mostly conferred by enzymatic hydrolysis of the antibiotic, and this mechanism is used by different gram-negative bacteria including Enterobacteriaceae. The enzymes responsible for the hydrolysis are β-lactamases like OXA, CTX-M, TEM and AmpC (Iredell *et al.*, 2016; Xia *et al.*, 2016). Extended-Spectrum Beta-Lactamases (ESBLs) are enzymes that can be carried on bacterial plasmids and produced by different gram-negative

bacteria, especially by *E. coli* that cause UTIs, and they confer increased resistance to a large number of antibiotics. It has been established that the ESBL-related infections have played a significant role in worsening the issue of antibiotic resistance all across the globe (Davies and Davies, 2010; Cantón *et al.*, 2012; Winkler *et al.*, 2015). β-lactam antibiotics have been categorised into some groups based on the chemical structure of the β-lactam ring. Susceptibility to enzymatic hydrolysis is conferred by the β-lactam ring structure. The fused ring structure increases this susceptibility (Bush and Jacoby, 2010; Lewis, 2013).

Based on the Ambler scheme classification, TEM belongs to class A according to the nucleotide and amino acid sequences of TEM enzymes (Shapiro, 2017). More than 170 modified TEM-1 and TEM-2 derivatives currently have phenotypes which include resistance to second-generation cephalosporins like (Cefaclor) and third-generation cephalosporins like (Cephalexin), monobactams like (tigemonam), and to β-lactamase inhibitors (Bush and Jacoby, 2010; Salverda *et al.*, 2010). TEM is inhibited by clavulanic acid and can hydrolyse ampicillin more than carbenicillin, oxacillin, or cephalothin (Paterson and Bonomo, 2005). As of 2015, more than 130 TEM-type β-lactamases have been isolated (Bonnet, 2004; Bajaj *et al.*, 2016).

AmpC β-lactamases provide resistance to many penicillin antibiotics, as well as extended-spectrum cephalosporins (Jacoby, 2009). Cefoxitin resistance is used as an indicator of AmpC-mediated resistance. However, it can also be an indication of the loss of outer membrane permeability (Philippon *et al.*, 2002). The chromosomal *ampC* gene expression in these organisms is inducible by β-lactam antibiotics, such as cefoxitin and imipenem; however, it is poorly induced by third-generation cephalosporins or fourth-generation cephalosporins like (cefepime).

Oxacillin-hydrolyzing (OXA) β-lactamases belong to class D, according to Ambler (Ambler *et al.*, 1991). OXA β-lactamases attack oxyimino-cephalosporins and have a higher

hydrolytic activity against oxacillin, methicillin, and cloxacillin than against benzylpenicillin (Walther-Rasmussen and Høiby, 2006). Most OXA-type β-lactamases are not regarded as ESBLs because OXA does not hydrolyse extended-spectrum cephalosporins. The enzyme kinetics of OXA-48 shows a low level of hydrolytic activity against carbapenems and better activity with imipenem than with meropenem. OXA-48 contains a larger hydrophobic region in the active-site cleft, which allows meropenem's hydroxyethyl group better movement. Moreover, OXA-48 hydrolyses carbapenems at a low level but penicillins at a high level; consequently, OXA-48 producers may be either susceptible or resistant to broad-spectrum cephalosporins and carbapenems (Evans and Amyes, 2014).

The Polymerase Chain Reaction (PCR) is one of the most techniques in molecular biology. This technique is currently widely used by clinicians and researchers to diagnose diseases, clone and sequence genes, detection of pathogens, and carry out sophisticated quantitative and genomic studies in a rapid and very sensitive manner.

Two-dimensional gel electrophoresis (2-D gel) is a method for the analysis and separation of complex protein mixtures extracted from a microbe or other biological samples. 2-D gel technique consists of two steps, instituted to two independent properties. The first dimension is isoelectric focusing (IEF) that separates proteins according to their isoelectric points (pI). And the second dimension is SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) that separates proteins based on to their molecular weights (MW). In this method, the large mass range and complete amount of proteins can be analysed at one-time, high resolution of the protein by (pI) and molecular mass, Comparison between resolved proteins spots for different conditions and use them for mass spectrometry (MS) analysis (Aebersold and Cravatt, 2002; Zhu *et al.*, 2010).

Development of mechanisms of antibiotic resistance in bacterial cells is almost as old as the development of antibiotics that includes the development of β-lactams as well (Davies and Davies, 2010; Iredell *et al.*, 2016). Bacteria have devised different mechanisms of antibiotic resistance due to selective pressures intensified by unnecessary use of antibiotics. Determinants of resistance are chromosomal as well as extra-chromosomal. More importantly, mobilisation of these determinants is possible through transformation, transduction and conjugation. Antibiotic resistance has spread among bacteria all across the globe mostly because of the exchange of mobile resistance determinants occurring mostly through transmission of plasmids (Davies and Davies, 2010). Several different mechanisms of antibiotic resistance have been evolved by gram-negative as well as gram-positive bacteria (Iredell *et al.*, 2016).

Since 1980, β-lactam antibiotics have been widely used for the treatment of infections, especially with gram-negative bacteria. ESBLs confer resistant to penicillins, first-, second-, and third-generation cephalosporins. Clavulanic acid inhibits aztreonam, but through the hydrolysis of these antibiotics (Paterson and Bonomo, 2005; Rawat and Nair, 2010a). In 2011, the European Centre for Disease Control and Prevention (ECDC) stated that the spread of ESBL +ve *E. coli* had increased in more than half of the 28 European countries over the previous four years (Beytur *et al.*, 2015).

In this chapter, bacterial strains isolated from patients diagnosed with UTI were identified using polymerase chain reaction analysis. Also, to characterise ESBL+ve and –ve *E. coli* strains cellular and extracellular protein profiles by SDS-PAGE and 2-D gel electrophoresis, and their antimicrobial susceptibility patterns were explored.

2.2 Materials and methods

All the chemicals for this study were purchased from Sigma Laboratories and Thermofisher unless otherwise stated.

2.2.1 Bacterial culture

Four clinical strains of *E. coli* (ESBL +ve (TEM, AmpC, and OXA-48) and ESBL–ve as a control) were used in this study. They were isolated from patients with UTIs and provided by Dr Tony Elston from Colchester General Hospital. The bacterial strains were grown on Cysteine Lactose Electrolyte Deficient agar (CLED) plates. Before each experiment, bacteria were inoculated in Luria-Bertani (LB) medium and incubated at 37°C for 6h (log or exponential growth phase). Their optical density (OD) was measured in LB broth using a spectrophotometer (Cecil CE 2041) at a wavelength of 595nm and adjusted to give an OD of 0.22 (~ 10^8 CFU/ml).

2.2.2 Bacterial DNA extraction

Bacterial DNA was extracted according to the manufacturer's instructions (Qiagen 563044). After LB incubation, broths were collected in the eppendorf tubes and centrifuged for 10min at 5000 x g then, washed twice with (PBS) X1 and pellets were lysed in 180µl of tissue lysis buffer (ATL). Samples were placed in a water bath at 56°C for 4h. Then, 200µl of AL buffer were mixed with 1µl of carrier RNA (carrier RNA enhances binding of DNA to the QIAamp MinElute column membrane, especially if there are very few target molecules in the sample). The mixed solutions were vortexed, and 200µl of ethanol (95–100%) were added to the mixed solutions, and the resulting samples were mixed and left for 5min at RT. Samples were centrifuged and transferred to the QIAamp MinElute column and centrifuged for 1min at 8000 x g. About 500µl Buffer AW1 was added to the QIAamp MinElute column, and samples were

centrifuged for 1min at 8000 x g when 500µl Buffer AW2 was added to samples, and then centrifuged for 1min at 8000 x g. The tubes were centrifuged for 3min at 14,000 x g to dry the membrane and 20–100µl of Buffer AE were added to the mid-point of the membrane, and samples were placed on the bench for 5min at RT and then centrifuged for 1min at 14,000 x g. The final products contained the DNA that had been extracted from the bacteria. Usually, the final products were stored at -20^{o}C for one month.

2.2.3 Bacterial plasmid detaching

To extract the plasmid DNA from AmpC *E. coli* strain, QIAamp Spin Miniprep Kit was used. The bacteria were harvested by centrifugation at 3000 x g for 10min. The pelleted bacteria were re-suspended in 250µl of Buffer P1 and transferred to a micro-centrifuge tube before adding 250µl of Buffer P2 and gently inverting the tubes six times to mix the contents, and then continuing to invert the tubes until the solutions became viscous and slightly clear. However, the lysis stage should last for no longer than 5min. After that, 350µl of Buffer N3 were added, and the tubes were inverted immediately (but gently) six times to mix the contents so that the solutions became cloudy before centrifuging the contents for 10min at 13,000 x g in a tabletop microcentrifuge. The supernatants were applied to the QIAprep spin column by pipetting, centrifuged for 45s, and the flow-through was discarded. The QIAprep spin columns were washed by adding 0.75ml Buffer PE and centrifuged for 45s. Then the flow-through was discarded, and the samples were centrifuged for 1min. Finally, the QIAprep columns were placed in a clean 1.5-ml microcentrifuge tubes, 50µl Buffer EB (10 mM Tris·Cl, pH 8.5) were added to the centre of the QIAprep spin column, and the samples were centrifuged for 1min at 13,000 x g, which contains the plasmid DNA.

2.2.4 Bacterial protein extraction

To ascertain differences in the protein profiles of the bacterial strains, both the extracellular and intracellular proteins of the bacterial strains were extracted following which they were analysed. Bacterial strains were grown onto CLED plates at 37°C overnight. Then, bacteria were inoculated into LB broth and incubated at 37°C overnight. The next day, samples were centrifuged for 10min at 5000 x g. Then, supernatants were used to extract extracellular protein and pellets were used to extract intracellular proteins.

2.2.4.1 Bacterial extracellular protein extraction

The extracellular protein extraction was employed to study the extracellular proteases produced by different strains of *E. coli*. The culture supernatants (from bacterial broths) were passed through the filters with a diameter of 0.2µm and stored in 15ml tubes. 400µl of methanol, 200µl of chloroform, and 300µl of distilled water (dw) were added to the 500µl of sample filtrates. The samples were then shaken and centrifuged for 7min at 5000 x g. There were three layers; the top layer was carefully removed, and then 300µl of methanol were carefully added. The samples were centrifuged again at 5000 x g for 7min and the supernatant removed with the precipitated proteins then air dried and stirred after the addition of 25µl of SDS-sample buffer (Sci *et al*., 2000).

2.2.4.2 Bacterial intracellular protein extraction

To analyse cellular proteins, the pellets from the previous step were used, and 1ml of PBS was added in each sample, then samples were centrifuged again for 5min at 14,100 x g. After centrifugation, 1ml of lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 15 mM pH8.5 Tris) was added in each sample, and the mixtures were vortexed well then, samples were left for

15min at RT and centrifuged for 25min at 14,100 x g. Finally, supernatants were collected which contain the desired proteins for analysis (Zhou *et al.*, 2006).

2.2.5 Genomic analysis

2.2.5.1 Polymerase Chain Reaction (PCR)

To characterise the bacteria used in this study, specific primers (*E. coli* 16S rRNA, TEM, AmpC, OXA-48, CTX-M, and Usp - Table 2.1) were used in PCR to confirm the identities of the strains. Each PCR reaction was carried out in 50µl reaction volumes in the PCR tube by adding the following: Buffer 10X containing 5µl (KCl, Tris)-$MgCl_2$, 3µl $MgCl_2$, 1µl 10mM dNTPs, 1-10ng of reverse primer, 1-10ng of forward primer, 1µl DNA, 0.5µl Taq polymerase, and 38µl dw. The PCR cycling setup was different for each gene as listed in (Table 2.2). The PCR products were then kept at 4ºC for analysis. Using 0.5% agarose gels (The 0.5% agarose gels were prepared by dissolving 400mg of agarose in 40ml of TAE 1X buffer (4.84 g Tris base, 2ml 0.5M EDTA, and 1.14ml pH eight glacial acetic acid)). Gels were heated to the boiling point and left at RT to cool down then 5µl from safeview were added to the gel. The gel was placed in the system and samples were loaded after mixing with the sample-loading buffer (10mM Tris-HCL (pH7.6), 60% glycerol, 60mM EDTA) at 5:1. Gels were run constantly at 150 V for 15min, depending on the size of the observed fragments. Finally, gels were checked under the Ultraviolet light (UV).

Table 2.1 Gene and primer sequences for the phylogenetic grouping of *E. coli*.

Target gene	Primer (nucleotide sequence)	reference	Product length (bp)
E.coli 16S rRNA	F 5’ -CCCCCTGGACGAAGACTGAC- 3’	(Wang *et al.*, 2002)	450 bp
	R 5’ -ACCGCTGGCAACAAAGGATA- 3’		
TEM	F 5’-ATGAGTATTCAACATTTCCG-3´	(Chroma and Kolar, 2010; Kolar *et al.*, 2010)	850 bp
	R 5´-CCAATGCTTAATCAGTGAGC-3´		
AmpC	F 5' -GATCGTTCTGCCGCTGTG-3'	(Corvec *et al.*, 2002)	271-bp
	R 5' -GGGCAGCAAATGTGGAGCAA- 3'		
OXA-48	F 5′-TTGGTGGCATCGATTATCGG-3′	(Poirel *et al.*, 2012)	750 bp
	R 5′-GAGCACTTCTTTTGTGATG GC-3′		
CTX-M	F 5’ -ATG TGC AGY ACC AGT AAR GT-3’	(Ramadan *et al.*, 2016)	544 bp
	R 5’ -TGG GTR AAR TAR GTS ACC AGA- 3’		
Usp	F- 5’ - ACATTCACGGCAAGCCTCAG- 3’	(Johnson and Stell, 2000)	440 bp
	R- 5’ -AGCGAGTTCCTGGTGAAAGC- 3’		

Table 2.2 The setup of PCR cycling for the different genes under investigation.

E.coli 16S rRNA	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	08	00
Denaturation	95 ºC	00	30
Annealing	58 ºC	00	30
Extension	72 ºC	00	30
Final Extension	72 ºC	07	00
Cycles	30		
TEM	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	05	00
Denaturation	94 ºC	00	30
Annealing	55 ºC	00	60
Extension	70 ºC	00	60
Final Extension	75 ºC	10	00
Cycles	30		
AmpC	Temperature	Time	
		min	s
Initial Denaturation	94 ºC	10	00
Denaturation	94 ºC	00	30
Annealing	55 ºC	00	45
Extension	72 ºC	01	00
Final Extension	72 ºC	10	00
Cycles	30		
OXA-48	Temperature	Time	
		min	s
Initial Denaturation	94 ºC	05	00
Denaturation	94 ºC	00	60
Annealing	56 ºC	00	45
Extension	72 ºC	01	00
Final Extension	72 ºC	07	00
Cycles	35		
CTX-M	Temperature	Time	
		min	s
Initial Denaturation	94 ºC	05	00
Denaturation	94 ºC	00	45
Annealing	50 ºC	00	40

Extension	72 ºC	01	00
Final Extension	72 ºC	10	00
Cycles	32		
AmpC with plasmid	Temperature	Time	
		min	s
Initial Denaturation	ºC	10	00
Denaturation	ºC	00	30
Annealing	ºC	00	45
Extension	ºC	01	00
Final Extension	ºC	10	00
Cycles	30		
Usp	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	15	00
Denaturation	94 ºC	00	30
Annealing	58 ºC	00	30
Extension	72 ºC	01	00
Final Extension	72 ºC	05	00
Cycles	30		

2.2.5.2 Purification of PCR product

To further identify the isolates sequence and definition the strains, the PCR products were purified by using the QIAquick PCR purification kit using a microcentrifuge (QIAGEN Ltd., Crawley, UK). In brief, 5µl of the PCR products were mixed with 25µl of buffer PB. The mixture was further mixed with 10µl of 3M sodium acetate (pH 5.0), and when the colour of the mixtures turned yellow, they were applied to a QIAquick column and centrifuged at 17900 x g for 60s. The flow-through was discarded, the products were washed with 750µl of buffer PE and centrifuged at 17900 x g for the 60s. Again, the flow-through was discarded, and the QIAquick columns were centrifuged at 17900 x g for another 60s. Then, the QIAquick columns were placed in clean 1.5ml microcentrifuge tubes, and DNA was eluted by adding 30µl elution buffer

to the centre of the QIAquick membrane. The columns were allowed to stand at RT for 1min and centrifuged at 17900 x g for 60s.

2.2.5.3 Gene sequencing

To characterise the isolates, purified PCR products were commercially sequenced by using Sanger Sequencing Services (Source Bioscience Sequencin, Nottingham, UK). One-way of *E. coli* 16S (5’ -CCCCCTGGACGAAGACTGAC- 3’), TEM (5’ -ATGAGTATTCAACATTTCCG-3´), and OXA-48 (5’ -TTGGTGGCATCGATTATCGG- 3’) primers were used to obtain the sequences.

2.2.5.4 BLAST search and phylogenetic analysis

Sequences were identified by using the nucleotide Basic Alignment Search Tool (n-BLAST). Nucleotide collection database from GenBank was used, and the program selection was optimised for highly similar sequences (megablast). DNA quality check, and DNA sequencing was performed by FinchTV program (Mohamudha *et al.*, 2012). Phylogenetic analysis based on the sequences obtained was performed using MEGA with default settings and configured for highest accuracy and phylogeny.fr platform (Dereeper *et al.*, 2008). MUSCLE (v3.8.31) was used to align the sequences with default settings and configured for highest accuracy. Finally, the tree was graphically represented and edited with TreeDyn (v198.3).

2.2.6 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

To determine the protein profile of the three ESBL +ve and –ve strains of *E. coli*, SDS-PAGE was performed. All clinical strains were plated onto CLED agar plates and incubated at 37°C overnight. All bacteria were inoculated in LB medium broth and incubated at 37°C overnight. For every bacterial strain, the OD was measured at 595nm. The running buffer for the SDS-PAGE was prepared: 3gm Tris, 14.4gm glycine, and 1gm SDS in 1000ml of dw. Then, the sample buffer (0.06M Tris, 2.5% glycerol, 0.5% SDS, 1.25% 2-mercaptoethanol, and 0.001% bromophenol blue) (pH6.8) and 10% SDS-PAGE gel (4.2ml dw, 3.3ml Bis/acrylamide solution, 0.1ml 10% SDS, 2ml 1.5M pH 7.4 Tris, 400µl 10% ammonium persulphate, and 6µl Tetramethylethylenediamine (TEMED) solution) was prepared. Also, a 4% stacking gel (4.75ml dw, 0.75ml BIS/acrylamide, 1.88ml 0.5M pH 6.8 Tris, 75µl 10% SDS, 75µl 10% ammonium persulphate, and 3.2µl TEMED solution) was also prepared ready to run the samples. Electrophoresis was performed by using a Bio-Rad mini-protein apparatus with a vertical slab gel of 7cm (L) x 8cm (W) x 0.75mm (T). The concentration of the bacterial extract was determined in a Bradford protein assay following which sample buffer was added to the extracts before loading on the gel. The gel was run at a constant voltage of 100 V for 45min, and the voltage was then increased to 150 V. Then, the gel was stained using a silver staining method (Bio-Rad, UK). After running, the gel was placed in the fixative enhancer solution (50% methanol, 10% acetic acid, 10% fixative enhancer, and 30% dw) with gentle agitation for 20min at RT on the shaker. Then, the fixative enhancer solution was removed, and the gel was washed twice with dw for 10min. The gel was stained with silver staining solution (35ml deionised water, 5ml silver complex solution, 5ml reduction moderator solution, 5ml image development reagent, and 50ml development accelerator solution) until the desired staining intensity was reached. Finally, the reaction was stopped by adding stop solution (5% acetic acid solution) for 15min. After that, the gel was washed with water for 5min and photographed.

2.2.7 Two-dimensional gel electrophoresis (2D Gel)

2D gel electrophoresis is another tool for the analysis of complex protein mixtures extracted (Intracellular protein and extracellular protein) from bacteria (Chevallet *et al.*, 2006). This process involves the followings:

2.2.7.1 First dimensional gel isoelectric focusing (IEF)

The immobiline dry strip gel (IPG) (11cm, pH 4-7) (Bio-Rad, UK) was rehydrated in an immobiline dry strip reswelling tray, following the outlined protocol by (Bio-Rad, UK). The dry strip was rehydrated for 24h at RT with the sample proteins (after measuring the protein concentrations via the Bradford protein assay). The samples were dissolved in 300µl of rehydration buffer (7M urea, 2M thiourea, 4% CHAPS, 1X protease inhibitor cocktail, 20mM DL-Dithiothreitol (DDT), 1% ampholyte, 0.1% bromophenol blue, and 0.05% SDS). After rehydration, the IPG strip was gently rinsed with dw 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 an immobiline PlusOne dry strip to cover the fluid, and the rehydrated strip was then placed in individual lanes of the Ettan IPG strip holder. The lid was closed, and the IPG-Phor program was run according to the program instructions. At the end of the program, the IPG strip was placed in a petri dish, rinsed with dw, labelled, and stored at –80^{o}C for a maximum of one year.

2.2.7.2 Second SDS-PAGE gel preparation

For the second-dimensional gel electrophoresis, the IPG strip was incubated at RT for 15min with 10ml of equilibration buffer (6M urea, 0.375M pH 8.8 Tris-HCl, 4% SDS, 20% glycerol, H_2O, and phenol red) containing 100mg of Dithiothrietol (DTT). Then, the strip was

incubated for 15min at RT with 10ml of equilibration buffer containing 400mg of iodo-acetamide. The IPG strip was rinsed with 1X electrophoresis buffer (3gm Tris, 15gm glycine, 10ml 10% SDS, and dw up to 1L) before placing it on the second-dimensional gel. The IPG strip was placed into the well containing 12% SDS-PAGE gel and sealed with agarose sealing solution, avoiding any air bubbles. Electrophoresis was carried out first at 50 V for 30min and then at 100 V till completion. Then, the gel was fixed with a gel-fixing solution (50% ethanol, 12% acetic acid, and 0.05% formalin).

For visualisation of protein spots, a modified silver staining protocol was constructed (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 with dw twice. Staining was done with 0.2% silver nitrate solution for 20min, followed by a careful wash twice with dw for a maximum of 1min each time. The gel was developed with 6% sodium carbonate and 0.0004% sodium thiosulfate for 3min or until spots appeared. Finally, the reaction was stopped by adding 12% acetic acid and shaking it for 10min. The gel was stored in 5% acetic acid at 4°C and scanned using the scanner (Epson image scanner III) with LabScan 6.0 software. The gel images were saved as MEL and TIFF files.

2.2.8 Susceptibility testing by disc diffusion (EUCAST Method)

Several antibiotics were used to determine bacterial sensitivity testing. They were gentamicin (CN/10µg), ampicillin (Amp/10µg), clavulanic acid- amoxycillin (AMC 2/10µg), ceftazidime (CAZ/30µg), imipenem (Ipm/10µg), ciprofloxacin (Cip 5mcg), cefotaxime (Acg 5mcg), and cefoxitin (FOX/30µg) (EUCAST, 2017). Briefly, the bacterial strains were grown on CLED agar plates at 37°C overnight. A few colonies were inoculated in LB broth and incubated at 37°C for 6h. The OD was measured to give $1x10^8$ bacteria /ml. Mueller-Hinton agar plates

(2.0g beef extract, 17.5g casein hydrolysate, 1.5g starch, 17.0g agar, and dissolved in 1L of dw) were inoculated, discs were applied, and plates were incubated at 37°C overnight (16-20h).

2.2.9 Susceptibility testing by minimum inhibitory concentration (MIC) (E-Test)

Antibiotic strips were used to determine the Minimum Inhibitory Concentration (MICs) of Amoxycillin/clavulanic acid (AMC 256µg/ml–0.015µg/ml) against bacteria under test (EUCAST, 2017). A few colonies were inoculated in LB and incubated at 37°C for 6h. The OD was measured to give $1x10^8$ bacteria /ml. Mueller-Hinton agar was inoculated, antibiotic strips were applied, and plates were incubated at 37°C overnight.

2.3 Results

2.3.1 Polymerase Chain Reaction (PCR)

Six sets of primers (*E. coli* 16S rRNA, TEM, AmpC, OXA-48, CTXM, and Usp) were used to amplify the *E. coli* 16s rRNA, TEM, AmpC, OXA-48, and Usp genes. PCR amplification was carried out on the chromosomal DNA of the clinical strains of *E. coli*. Additionally, PCR amplification of AmpC was carried out on plasmid DNA. The ESBL −ve *E. coli* was used as a negative control. All strains (*E. coli*-ve, TEM, Ampc, and OXA-48) were confirmed as bacteria when using 16S rRNA primer, as described in (Figure 2.1). The PCR products from the 16S rRNA of all bacteria were observed, and corresponded to the expected results, with approximately 450 bp being successfully amplified. The PCR products from the TEM, AmpC, and OXA- 48 were observed, and corresponded to the expected results, with approximately 850 bp, as described in (Figure 2.2). On the other hand, for ESBL –ve *E. coli*, no band was observed. To confirm the specificity of PCR product, OXA-48 primer was used with all bacteria under testing. For the ESBL –ve *E. coli*, TEM, and AmpC strains, no products were observed. On the other hand, only a PCR product from OXA-48 corresponded to the expected result, with approximately 750 bp was successfully amplified, as described in (Figure 2.3). Gel analysis of products following PCR with the CTXM primer showed that the expected 544 bp band was absent in both the TEM, AmpC and ESBL-ve strains but was present in the OXA-48 strain (Figure 2.4). All strains of *E. coli-ve*, TEM, and AmpC except OXA-48 were secreted uropathogen specific proteins when using Usp primer, as described in (Figure 2.5). On the other hand, gel analysis of the products from the PCR amplification of the *Usp* gene showed that the said gene was present in ESBL –ve *E. coli*, TEM, and AmpC strains, i.e. DNA band corresponded to the expected approximately 440 bp product. To find the relationships between these strains, a phylogenetic tree was obtained. By using three controls, TEM, OXA-48 and AmpC as well as the strains under testing. Figure 2.6 showed the phylogenetic tree generated by

the MEGA program with default settings and configured for highest accuracy and phylogeny.fr platform. Based on this phylogenetic tree, *E. coli* strains were grouped in 2 clusters. Interestingly, *E. coli* strains from phylogenetic groups based on ESBL+ve were clustered within the same phylogenetic groups. ESBL+ve from the phylogenetic group (B) were divided into 2 different clusters in the constructed tree. ESBL-ve isolate, however, belonged to a phylogenetic group (A) separate from that of the ESBL+ve isolates.

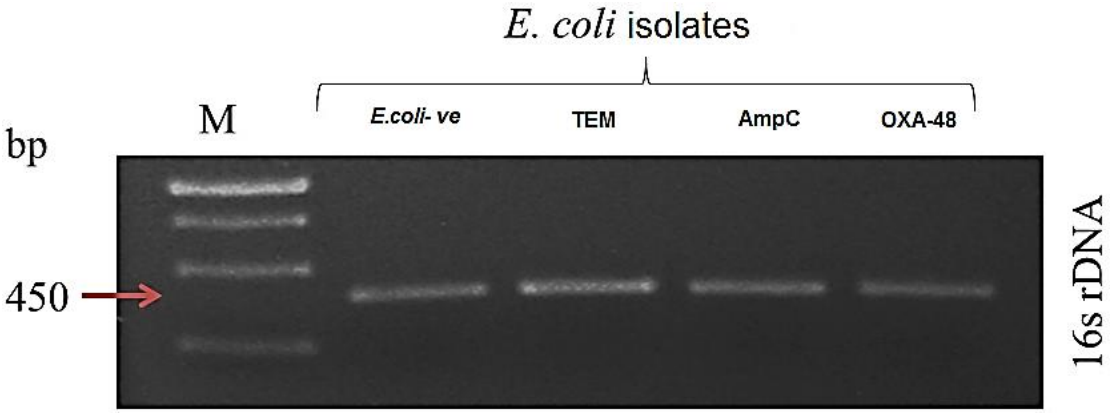

Figure 2.1: PCR products amplified using 16S rRNA primer. Bands were observed in all lanes with corresponded near to the expected approximately 450 bp. M – bp DNA ladder.

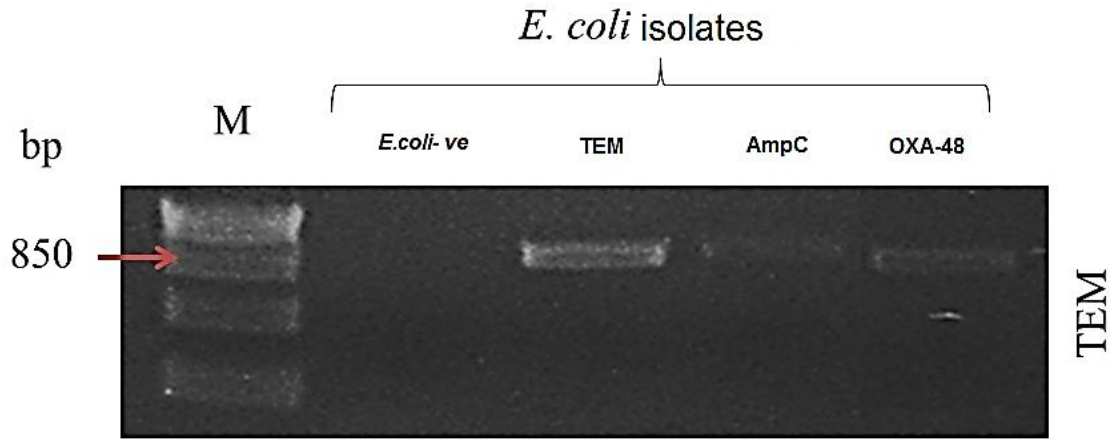

Figure 2.2: PCR products amplified using TEM primer. Bands were observed in TEM, Ampc, and OXA 48 corresponded near to the expected approximately 850 bp. M – bp DNA ladder.

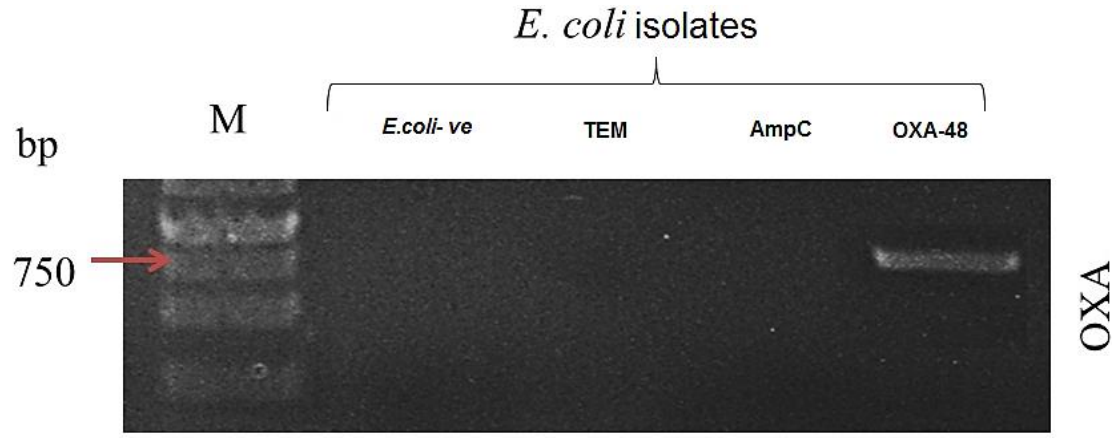

Figure 2.3: PCR products amplified using an OXA-48 primer. The band was observed in OXA-48 corresponded near to the expected approximately 750 bp: M – bp DNA ladder.

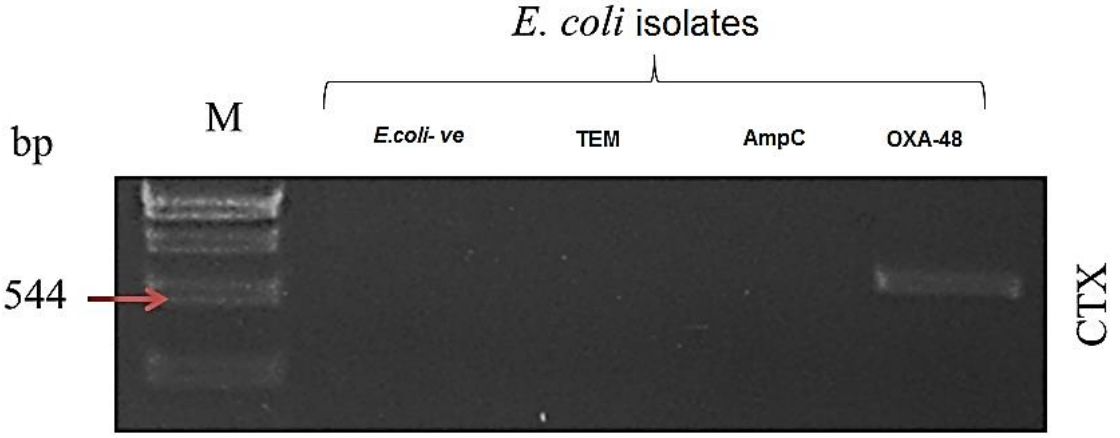

Figure 2.4: PCR products amplified using CTX M primer. The band was observed in OXA-48 corresponded near to the expected approximately 544 bp. M – bp DNA ladder.

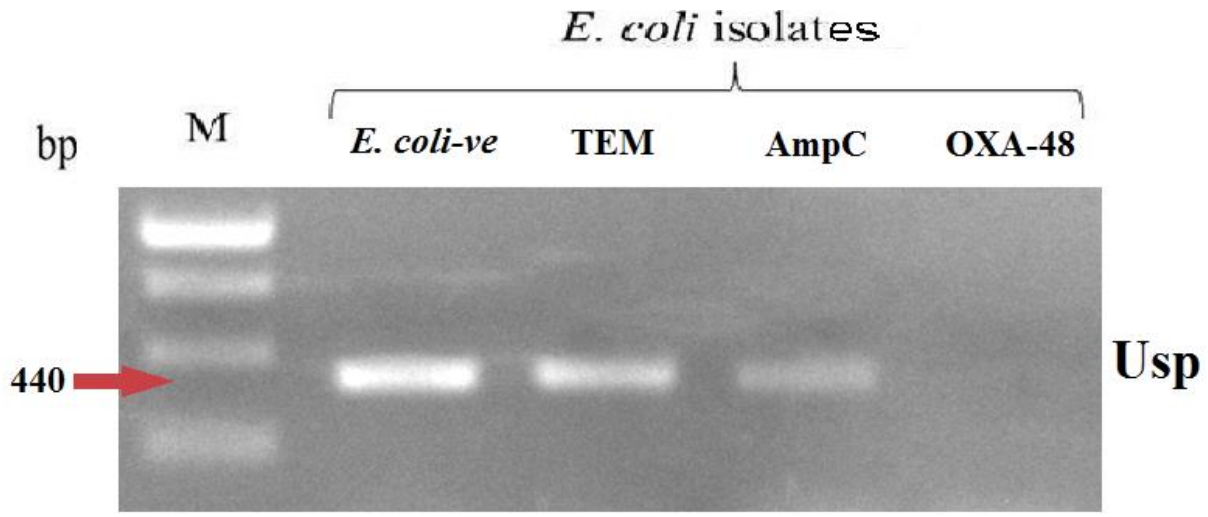

Figure 2.5: PCR products amplified using Usp primer. Bands were observed in *E. coli-ve*, TEM, and Ampc corresponded near to the expected approximately 440 bp. M – bp DNA ladder.

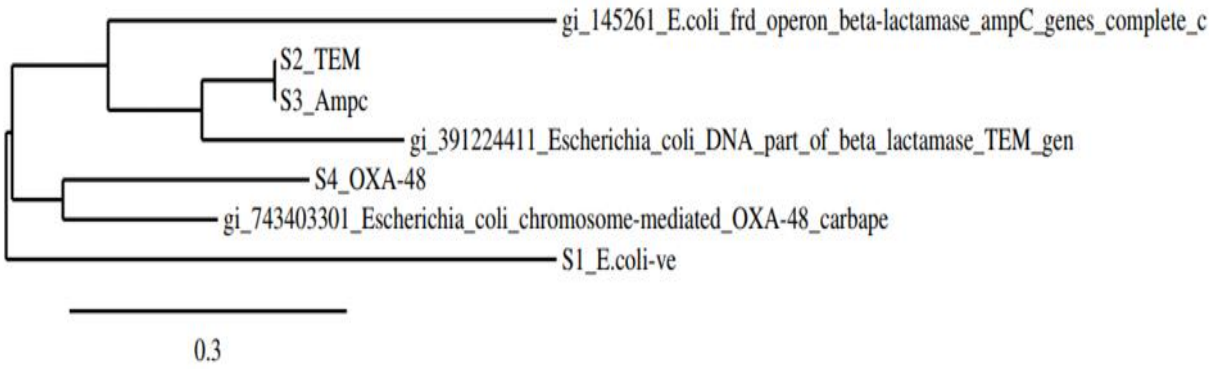

Figure 2.6: The phylogenetic tree of comparison of all samples (TEM, AmpC, and OXA-48) and the three positive controls used as reference sequences (391224411 is TEM, 743403301 is OXA-48, and 145261 is AmpC). *E. coli* strains were grouped in 2 clusters. ESBL+ve from the phylogenetic group (B) were divided into 2 different clusters in the constructed tree. ESBL-ve isolate belonged to a phylogenetic group (A) separate from that of the ESBL+ve isolates.

To determine the *AmpC* gene PCR amplification with the 16s rRNA primer was carried out on plasmid DNA, the product was observed. As expected, the results showed an approximate band of 450 bp (Figure 2.7). PCR amplification with the AmpC primer was carried out on plasmid DNA. As expected, the results showed an approximate band of 270 bp (Figure 2.8).

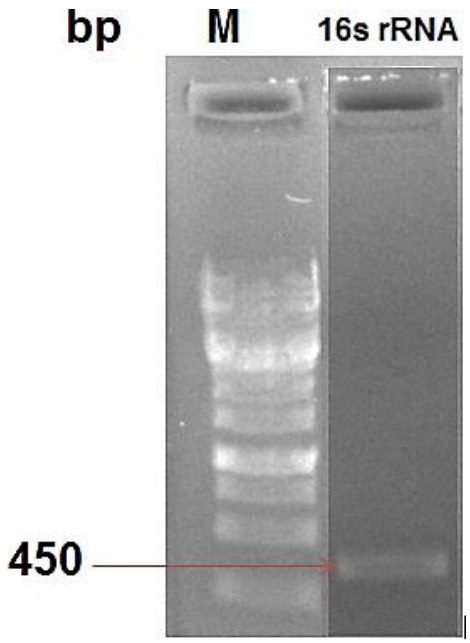

Figure 2.7: The AmpC strain with 16S rRNA primer, with PCR product approximately 450 bp. M – bp DNA ladder.

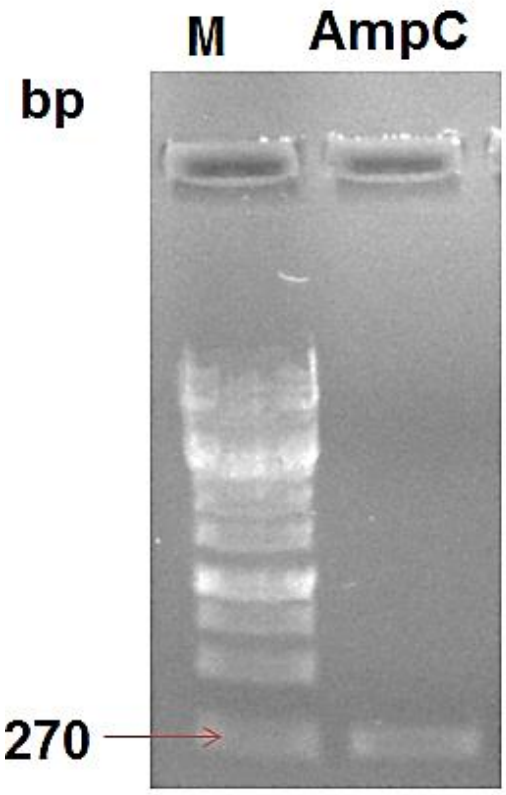

Figure 2.8: The AmpC strain with AmpC primer. The PCR product from AmpC corresponded to the expected result of approximately 270 bp. M – bp DNA ladder.

2.3.2 Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

In this study, SDS-PAGE was used to further the characterising bacterial protein profiles and to indicate the differences between the clinical strains. In addition, the procedure was employed to study the extracellular proteases produced by different strains of *E. coli*.

All strains showed similar protein profiles when using SDS-PAGE. However, there were a few differences between them as shown in (Figure 2.9). The comparison of the proteins in conditioned medium of all strains of *E. coli* showed nearly a close correspondence. Several differences can be seen in the patterns of the outer membrane proteins of all strains of *E. coli*, for example, there were many bands seen for the TEM strain, such as the ones at ≈ 60 KDa and ≈ 17 KDa.

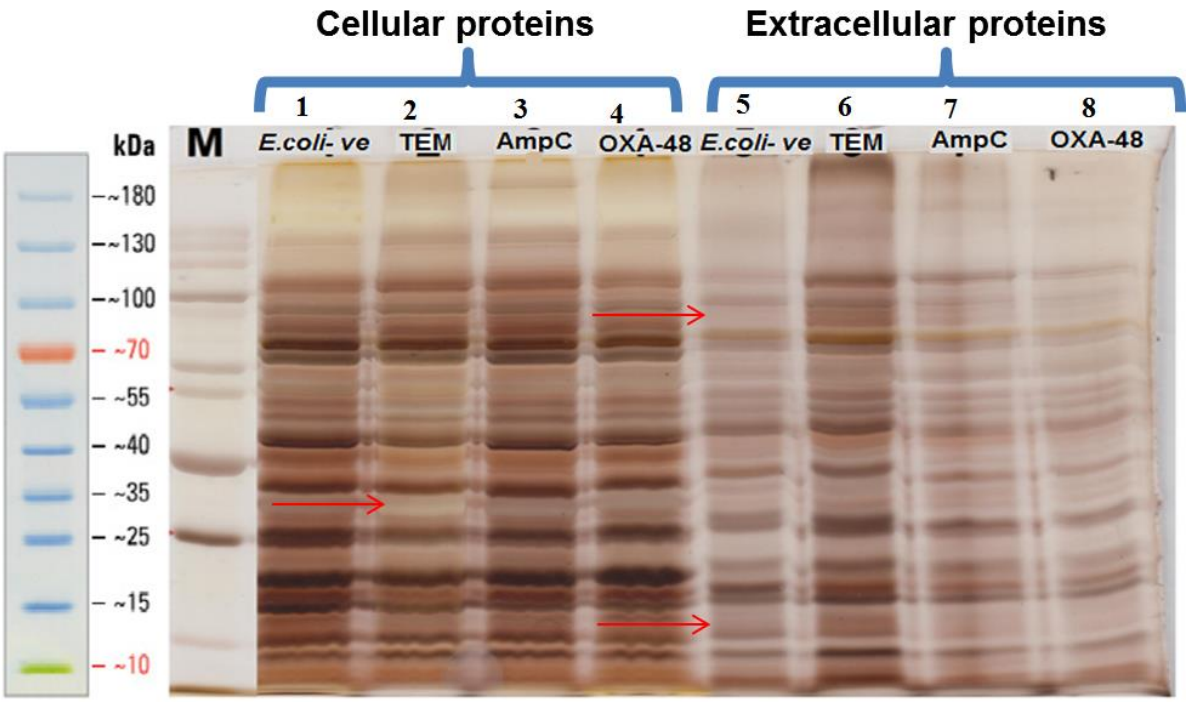

Figure 2.9: Protein profile of *E. coli* strains by SDS-PAGE.

M: Marker. Lanes 1-4 cellular proteins (1- (*E. coli-ve)* ESBL –ve, 2- TEM, 3- AmpC and 4- OXA-48). Lanes 5-8 Extracellular proteins in Bacterial Conditioned Media (5- (*E. coli-ve*) ESBL –ve strain, 6- TEM, 7-AmpC and 8- OXA-48). The red arrow indicates some differences in the appearance or absence of the bands.

2.3.3 Two-dimensional gel electrophoresis (2D Gel)

2.3.3.1 Extracellular protein extraction

In this study, 2D gel electrophoresis was used to study the extracellular proteases produced by different strains of *E. coli*. In this technique, every spot is scattered according to its molecular weight and pH, which is very helpful in identifying the protein. The total proteome was separated by first-dimension isoelectric focusing on the proteins' isoelectric points (pI). Then, the isoelectrically focused proteins were resolved by second dimension SDS-PAGE. Silver-stained protein spots in all are shown in Figure 2.10. The results of this proteomic analysis revealed the resolution of some polypeptide spots. Forty-eight protein spots were resolved for a

given pH (pI) and molecular weight (MW), as seen in (Figure 2.10). Gel images from all strains were then analysed using the Progenesis SameSpot software (Nonlinear Dynamics Limited), where the control (*E.coli*-ve) was used as a reference to find out differentially expressed proteins (Figure 2.11) (Table 2.3). Differentially expressed protein spots are circled with a purple colour, as seen in Figure 2.11. The results showed that many spots featured in AmpC (65.25% positive spots) (Figure 2.12). In addition, some spots featured in OXA-48 (54.16% positive spots) and TEM (52.08% positive spots). Moreover, there are many spots absent in ESBL –ve *E. coli* (41.66% positive spots), as shown in Table 2.4, the negative spots highlighted in red. Further analysis with 2D gel electrophoresis is needed to identify the sequence and identity the differentially regulated polypeptide spots that were observed.

Figure 2.10: Representation of a reference image showing all differentially expressed extracellular proteins of *E. coli* (ESBL-ve). The isoelectric points pI range is shown on the top, while the second-dimension SDS-PAGE is represented by an arrow on the left.

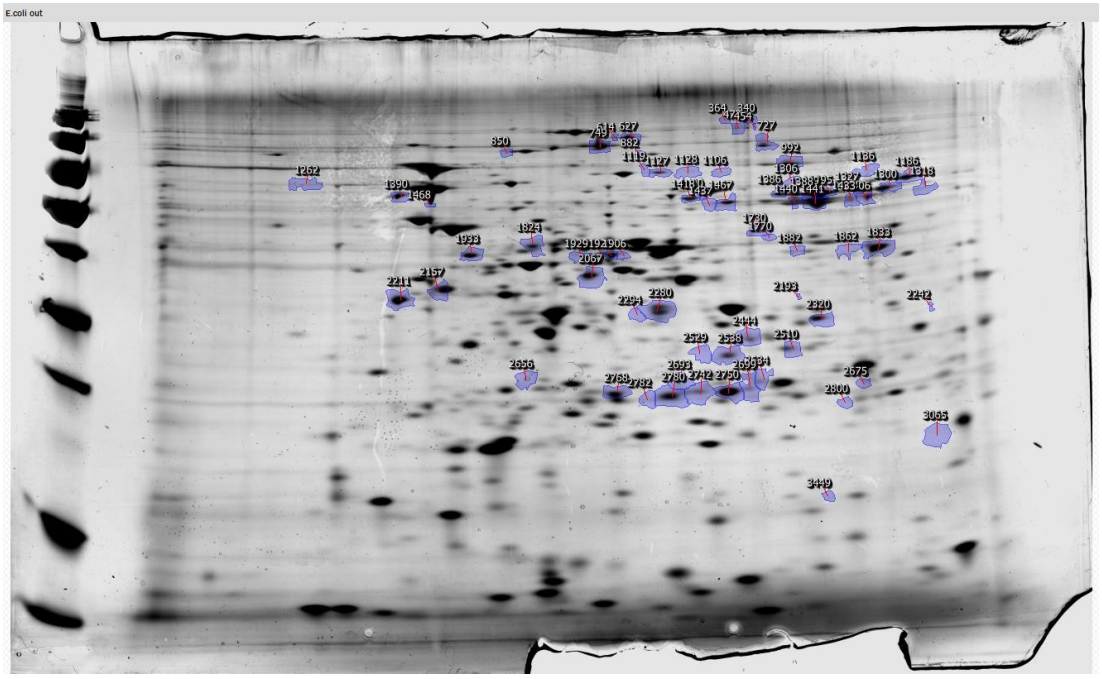

Figure 2.11: Representation of reference image showing all differentially expressed spots after analysis extracellular of proteins for *E. coli* (ESBL-ve) (spots circled with the purple colour). The reference image used for image analysis that was carried out with Progenesis SameSpot software.

Table 2.3 The Forty-eight specific protein spots identified by pI and MW among the clinical strains.

Spot number	pI (pH)	MW (kDa)	Spot number	pI (pH)	MW (kDa)
340	6.15	170	**364**	6.08	175
454	150	6.11	**473**	6.08	170
627	5.80	125	**749**	5.70	120
882	5.80	125	**1106**	6.08	83
1119	5.83	110	**1127**	5.87	90
1128	5.90	90	**1136**	5.53	98
1262	4.70	72	**1295**	6.40	85
1300	6.60	85	**1318**	6.70	85
1327	6.45	90	**1386**	6.23	75
1389	6.33	80	**1400**	6	80
1406	6.5	73	**1418**	5.96	72
1437	6.05	67	**1440**	5.28	71
1441	6.37	70	**1467**	6.10	68
1468	5.13	65	**1770**	6.21	54.5
1833	6.55	53	**1862**	6.44	50
1929	6.65	52	**2067**	5.70	41
2157	5.20	38.5	**2193**	5.32	39

2242	6.75	35.5	**2294**	5.84	37.5
2510	6.35	35	**2529**	6.07	34
2538	6.18	33.5	**2634**	6.28	31
2656	5.46	30	**2675**	6.68	31
2693	6.02	30	**2750**	6.15	29
2782	5.87	27	**2800**	6.51	27
3065	6.80	24	**3449**	6.45	19

Table 2.4 the spot numbers identified with the Progenesis Same Spot software. The results showed that the appearance of positive-signal spots is black and absent negative-signal spots are red.

Spots number	*E.coli* - ESBL	TEM	AmpC	OXA-48
340	Spot-	Spot-	Spot+	Spot-
364	Spot-	Spot-	Spot+	Spot-
454	Spot-	Spot+	Spot+	Spot-
473	Spot-	Spot+	Spot+	Spot-
627	Spot+	Spot+	Spot-	Spot+
749	Spot+	Spot+	Spot-	Spot+
882	Spot-	Spot-	Spot+	Spot-
1106	Spot-	Spot-	Spot-	Spot+
1119	Spot+	Spot-	Spot+	Spot-
1127	Spot+	Spot+	Spot+	Spot-
1128	Spot-	Spot+	Spot-	Spot+

1136	Spot-	Spot+	Spot+	Spot-
1262	Spot-	Spot-	Spot-	Spot+
1295	Spot+	Spot-	Spot+	Spot+
1300	Spot+	Spot+	Spot-	Spot+
1318	Spot-	Spot-	Spot+	Spot-
1327	Spot+	Spot+	Spot-	Spot+
1386	Spot+	Spot+	Spot+	Spot-
1389	Spot+	Spot+	Spot-	Spot+
1400	Spot-	Spot-	Spot+	Spot-
1406	Spot+	Spot+	Spot-	Spot+
1418	Spot+	Spot+	Spot-	Spot+
1437	Spot-	Spot-	Spot+	Spot+
1440	Spot+	Spot+	Spot-	Spot+
1441	Spot+	Spot+	Spot-	Spot+
1467	Spot+	Spot+	Spot-	Spot+
1468	Spot-	Spot-	Spot+	Spot-
1770	Spot-	Spot-	Spot+	Spot-
1833	Spot+	Spot+	Spot-	Spot+
1862	Spot-	Spot-	Spot+	Spot-
1929	Spot-	Spot+	Spot-	Spot+
2067	Spot+	Spot+	Spot-	Spot+
2157	Spot+	Spot+	Spot+	Spot-
2193	Spot-	Spot+	Spot+	Spot-
2242	Spot-	Spot-	Spot-	Spot+

2294	Spot-	Spot-	Spot-	Spot+
2510	Spot+	Spot+	Spot+	Spot-
2529	Spot-	Spot-	Spot-	Spot+
2538	Spot+	Spot+	Spot+	Spot-
2634	Spot-	Spot-	Spot+	Spot+
2656	Spot-	Spot+	Spot+	Spot+
2675	Spot-	Spot-	Spot+	Spot-
2693	Spot-	Spot-	Spot-	Spot+
2750	Spot+	Spot+	Spot+	Spot-
2782	Spot-	Spot-	Spot-	Spot+
2800	Spot-	Spot-	Spot+	Spot-
3065	Spot-	Spot-	Spot+	Spot+
3449	Spot-	Spot-	Spot+	Spot-
Number of positive spots	20	25	27	26
Number of negative spots	28	23	21	22

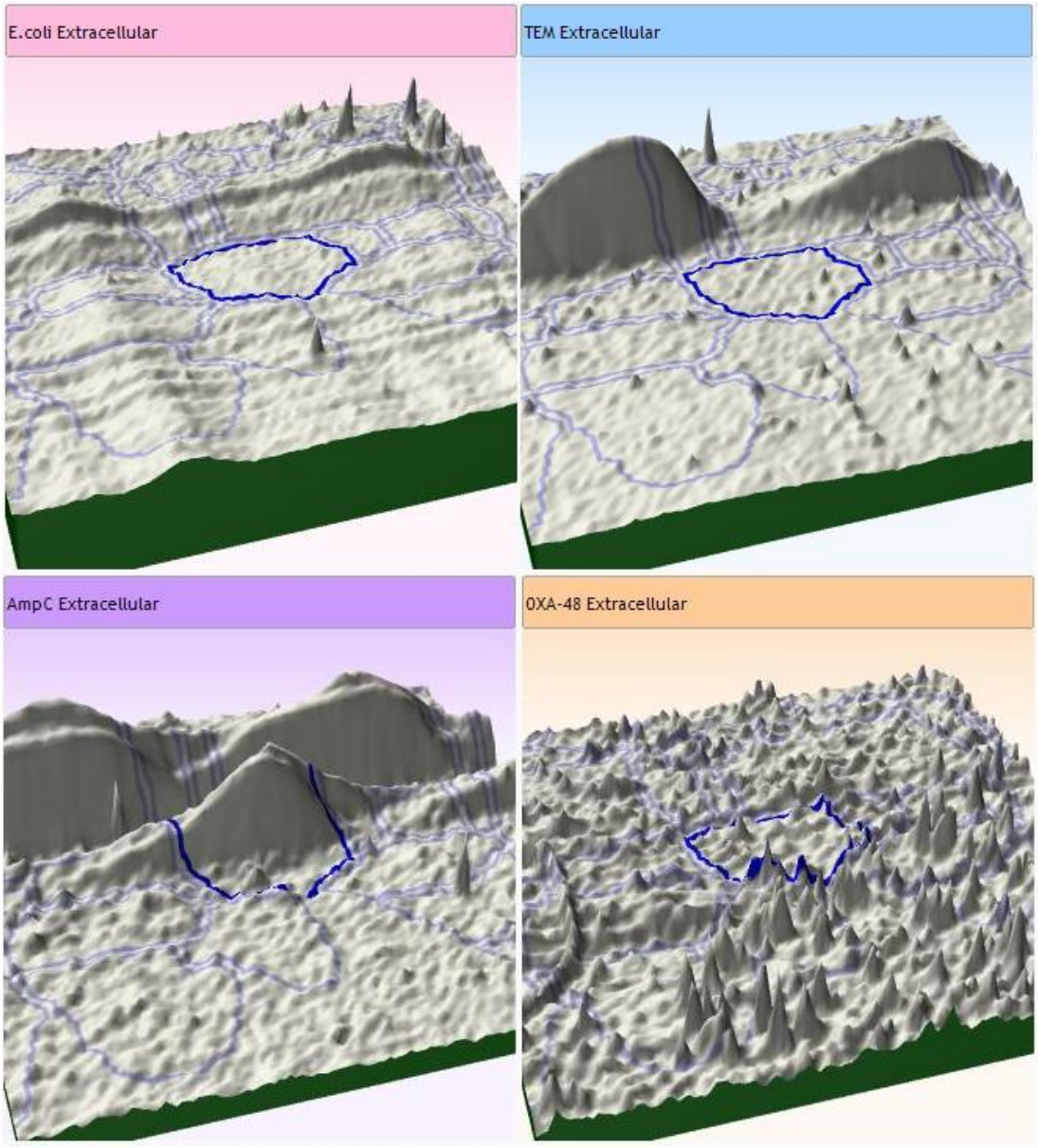

Figure 2.12: The 3D representations of spots identified by the Progenesis SameSpot software.

The results show spots in (AmpC strain) or down-regulated (E. coli-ve, TEM, and OXA-48 strains) between different strains.

2.3.3.2 Intracellular protein extraction

In this experiment, 2D gel electrophoresis was used to study the somatic proteins of *E. coli* strains. The results of this proteomic analysis revealed the resolution of some polypeptide spots. Forty-four protein spots were resolved for a given pH (pI) and molecular weight (MW), as seen in (Figure 2.13). Gel images from all strains were then analysed using the Progenesis SameSpot software (Nonlinear Dynamics Limited), where the control (*E. coli*-ve) sample gel was used as a reference to find out differentially expressed proteins (Figure 2.14) (Table 2.5). Differentially expressed protein spots are circled with a purple colour, as seen in (Figure 2.14). The results showed that many spots featured in OXA-48 (56.82% positive spots) (Figure 2.15). Also, some spots featured in TEM (34.10% positive spots) and AmpC (29.55% positive spots). On the other hand, there are many spots absent in ESBL –ve *E. coli* (27.27% positive spots), as shown in Table 2.6, the negative spots highlighted in red.

Uniprot was used to identify the proteins featured in all ESBL strains but absent in ESBL -ve *E. coli* strains. The polypeptide spot number 932 correlates with the probable Fe (2+)-trafficking protein (pI of ~5.71 and MW of ~110 kDa), which could be a mediator in iron transactions between iron acquisition and iron-requiring processes, such as synthesis and/or repair of Fe-S clusters in biosynthetic enzymes. Additionally, the spot number 1642 could be related to the phosphatidylserine decarboxylase proenzyme that catalyses the formation of phosphatidylethanolamine (PE) from phosphatidylserine at a pI of ~6.1 and has MW of ~60 kDa. The spot number 1734 with a pI of ~5.25 and MW of ~55 kDa could be related to the putative uncharacterised protein DDB_G0284813. The spot number 2460 could be related to the conotoxin LeD51, which has a pI of ~6.15 and an MW of ~37 kDa and plays a role in toxin and ion channel inhibitor activity. The spot number 2747, with a pI of ~5.38 and an MW of ~32 kDa, could be related to the conotoxin flf14c, which has toxin activity. Further analysis with 2D gel

electrophoresis is needed to identify the sequence and identity the differentially regulated polypeptide spots that were observed.

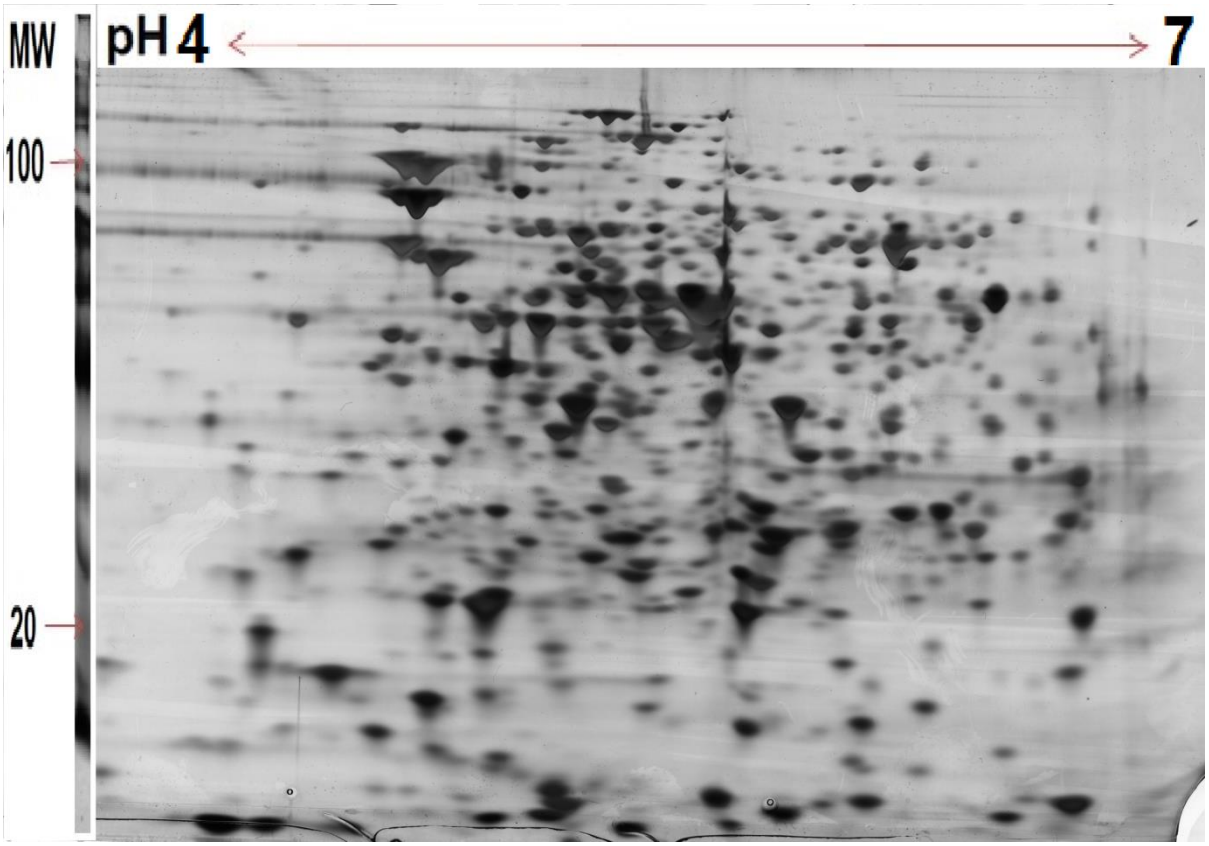

Figure 2.13: Representation of reference image showing all differentially expressed spot after analysis intracellular protein for *E. coli* (ESBL-ve). The reference image used for image analysis that was carried out with Progenesis SameSpot software.

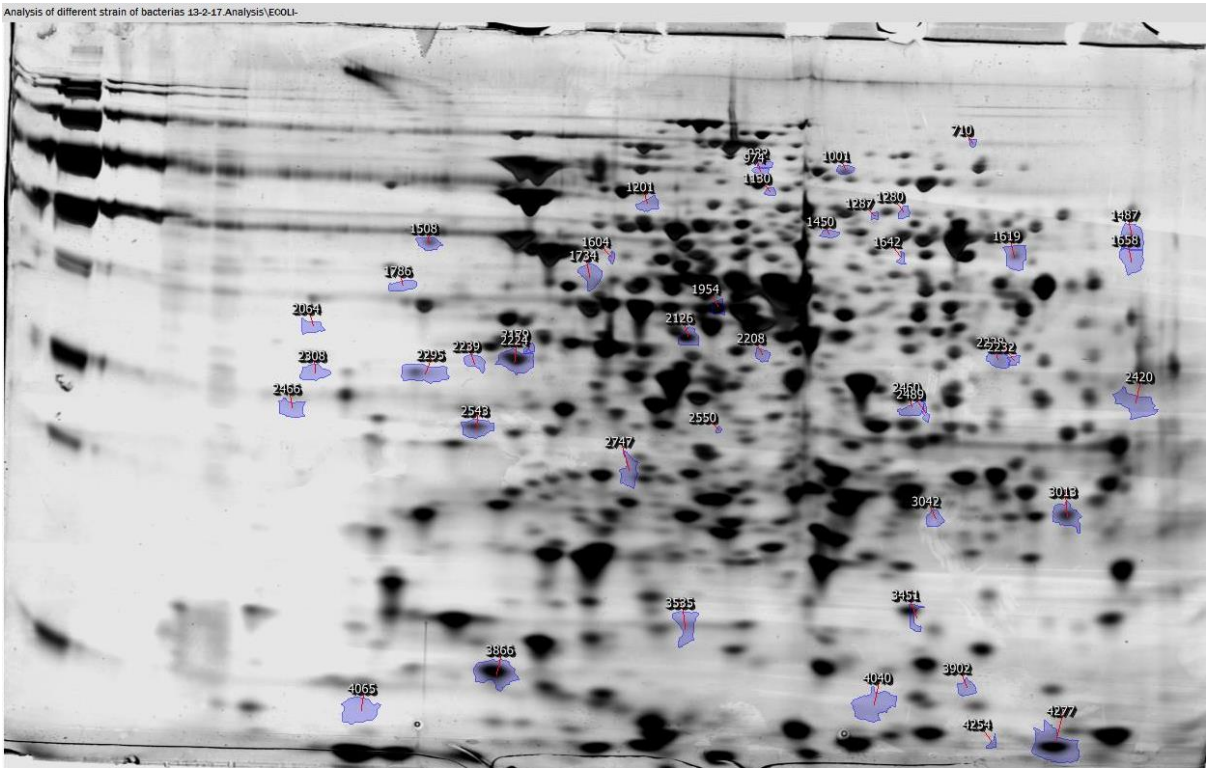

Figure 2.14: Representation of a reference image showing all differentially expressed intracellular proteins of *E. coli* (ESBL-ve) (spots circled with the blue colour). The isoelectric points pI range is shown on the top, while the second-dimension SDS-PAGE is represented by an arrow on the left.

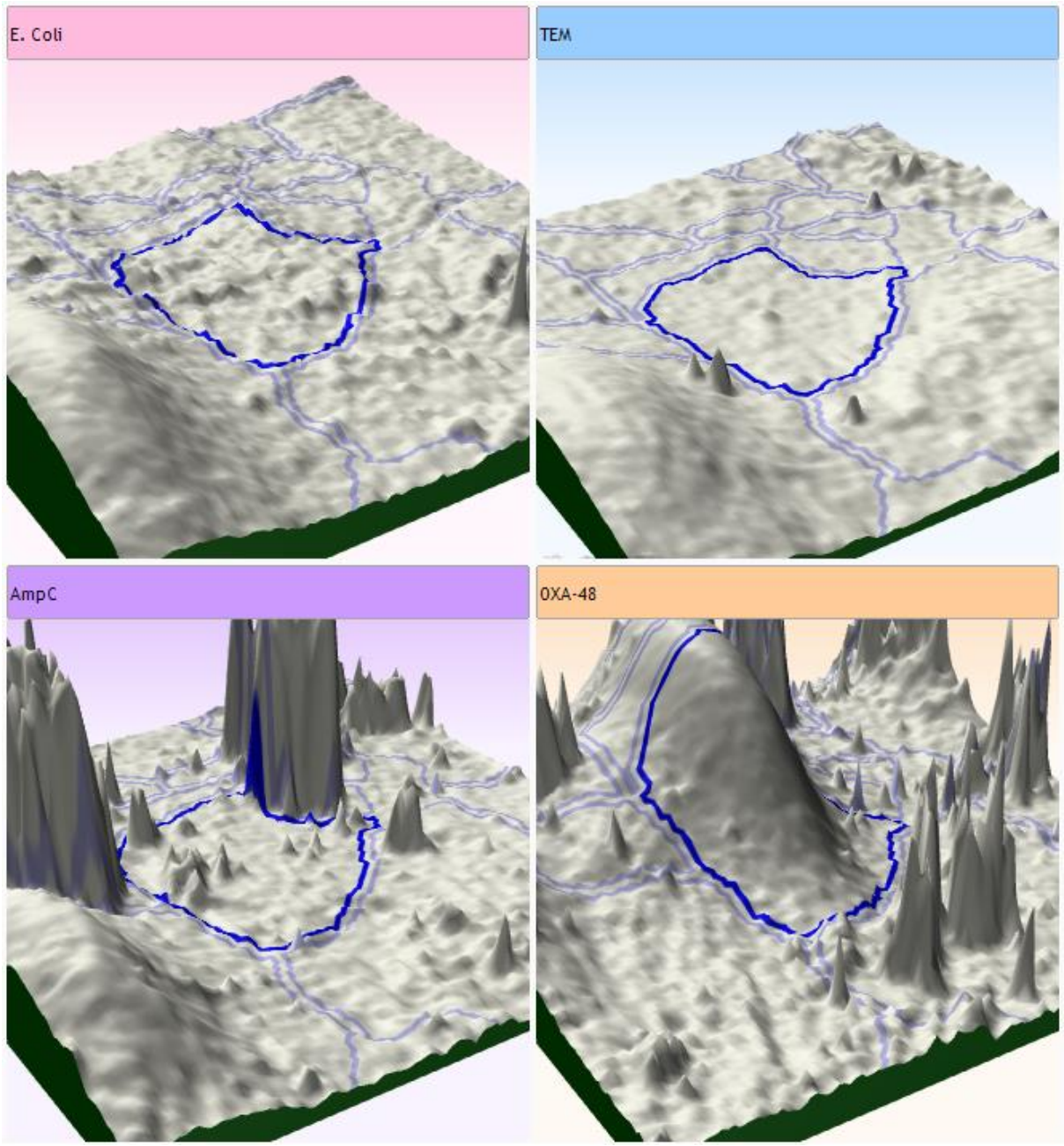

Figure 2.15: The 3D representations of spots identified by the Progenesis SameSpot software.

The results showed spots in (OXA-48 strain) or down-regulated (*E. coli*-ve, TEM, and AmpC strains) among different strains.

Table 2.5 The Forty-four specific protein spots identified by pI and MW between the clinical strains.

Spot number	pI (pH)	MW (kDa)	Spot number	pI (pH)	MW (kDa)
710	6.25	130	**932**	5.71	110
974	5.86	100	**1001**	5.9	100
1130	5.7	90	**1201**	5.40	80
1280	6.1	80	**1287**	6	77
1450	5.85	68	**1487**	6.65	67
1508	4.8	65	**1604**	5.30	61
1642	6.1	60	**1658**	6.68	60
1734	5.25	55	**1786**	4.75	53
1954	5.6	52	**2064**	4.55	48
2126	5.5	48	**2179**	5.1	43
2208	5.7	42	**2224**	5.05	42
2228	6.85	42	**2232**	6.80	42
2239	4.96	40	**2295**	4.78	40
2308	4.55	40	**2420**	6.70	38
2460	6.15	37	**2466**	4.3	37
2489	6.10	37	**2543**	4.9	36
2550	5.6	37	**2747**	5.38	32

3013	6.5	26	**3042**	6.15	26
3451	6.05	17	**3535**	5.49	15
3866	4.50	13	**3902**	6.23	13
4040	5.95	12.5	**4065**	4.65	12
4254	6.30	10.5	**4277**	6.45	10.5

Table 2.6 the spot numbers identified with the Progenesis Same Spot software. The results showed that the appearances of positive-signal spots are in black colour and absent negative-signal spots are red.

Spots number	*E.coli* - ESBL	TEM	AmpC	OXA-48
710	Spot-	Spot-	Spot-	Spot+
932	Spot-	Spot+	Spot+	Spot+
974	Spot-	Spot-	Spot-	Spot+
1001	Spot+	Spot-	Spot-	Spot-
1130	Spot-	Spot-	Spot+	Spot-
1201	Spot-	Spot+	Spot-	Spot+
1280	Spot-	Spot-	Spot-	Spot+
1287	Spot-	Spot-	Spot-	Spot+
1450	Spot-	Spot-	Spot-	Spot+
1487	Spot+	Spot-	Spot+	Spot-
1508	Spot+	Spot-	Spot-	Spot-
1604	Spot-	Spot+	Spot-	Spot-
1642	Spot-	Spot+	Spot+	Spot+
1658	Spot-	Spot-	Spot+	Spot-
1734	Spot-	Spot+	Spot+	Spot+
1786	Spot-	Spot-	Spot-	Spot+
1954	Spot+	Spot-	Spot-	Spot+
2064	Spot-	Spot-	Spot-	Spot+
2126	Spot+	Spot-	Spot-	Spot-

2179	Spot-	Spot+	Spot-	Spot-
2208	Spot-	Spot-	Spot-	Spot+
2224	Spot+	Spot+	Spot+	Spot-
2228	Spot-	Spot-	Spot-	Spot+
2232	Spot-	Spot-	Spot+	Spot+
2239	Spot-	Spot-	Spot-	Spot+
2295	Spot+	Spot+	Spot+	Spot-
2308	Spot-	Spot-	Spot-	Spot+
2420	Spot-	Spot-	Spot-	Spot+
2460	Spot-	Spot+	Spot+	Spot+
2466	Spot-	Spot-	Spot-	Spot+
2489	Spot-	Spot+	Spot-	Spot-
2543	Spot+	Spot-	Spot-	Spot-
2550	Spot-	Spot+	Spot-	Spot-
2747	Spot-	Spot+	Spot+	Spot+
3013	Spot+	Spot+	Spot+	Spot-
3042	Spot-	Spot-	Spot-	Spot+
3451	Spot+	Spot-	Spot-	Spot-
3535	Spot-	Spot-	Spot-	Spot+
3866	Spot+	Spot-	Spot-	Spot-
3902	Spot-	Spot-	Spot-	Spot+
4040	Spot-	Spot-	Spot-	Spot+
4065	Spot-	Spot+	Spot-	Spot-
4254	Spot-	Spot+	Spot-	Spot-
4277	Spot+	Spot-	Spot+	Spot-
Number of positive spots	12	15	13	25
Number of negative spots	32	29	31	19

2.3.4 Susceptibility testing by the disc diffusion method

The antimicrobial sensitivity using the EUCAST method for each of the clinical strains of *E. coli* was determined using the disc diffusion method (EUCAST 7, 2017). After overnight incubation, the diameter of inhibition zones around each antibiotic disc was measured as shown in Table 2.7 and Figures 2.16–2.18. The zone sizes obtained were compared with the zone diameter chart supplied by EUCAST (2017) and interpreted accordingly. This experiment showed that ESBL –ve *E. coli* strain was sensitive to all antibiotics except ampicillin, to which it was resistant, and gentamicin, with an intermediate sensitivity. TEM strain was sensitive to 70.0% of the studied antibiotic and resistant to only 25.0% (Figure 2.16). AmpC strain was sensitive to 50.0% and resistant to 37.5% of the studied antibiotics; it however exhibited intermediate sensitivity to gentamicin, (Figure 2.17). Finally, the OXA-48 strain was resistant to all antibiotics except ciprofloxacin, and it showed intermediate sensitivity to imipenem (Figure 2.18).

Table 2.7: susceptibility testing of ESBL +ve and –ve strains of *E. coli* against different antibiotics (Zone diameter breakpoint (mm)). S: sensitive, R: resistant, and I: intermediate.

Antibiotics	**Code/ Concentration (µg)**	***E. coli* –ve ESBL**		***E. coli* +ve ESBL (TEM)**		***E. coli* +ve ESBL (AmpC)**		***E. coli* +ve ESBL (OXA-48)**	
		Inhibition zone mm		Inhibition zone mm		Inhibition zone mm		Inhibition zone mm	
Gentamicin	CN / 10	15	I	10	R	15	I	0	R
Ampicillin	AMP / 10	13	R	0	R	0	R	0	R
Co-amoxiclave	AMC / 30	19	S	19	S	11	R	0	R
Ceftazidime	CAZ / 30	27	S	30	S	23	S	19	R
Imipenem	IPM / 10	28	S	31	S	29	S	21	I
Ciprofloxacin	CIP/ 5	29	S	28	S	31	S	26	S
cefotaxim	CTX/5	31	S	30	S	21	S	9	R
Cefoxitin	FOX / 30	26	S	24	S	15	R	15	R
% Resistance		12.5		25.0		37.5		75.0	

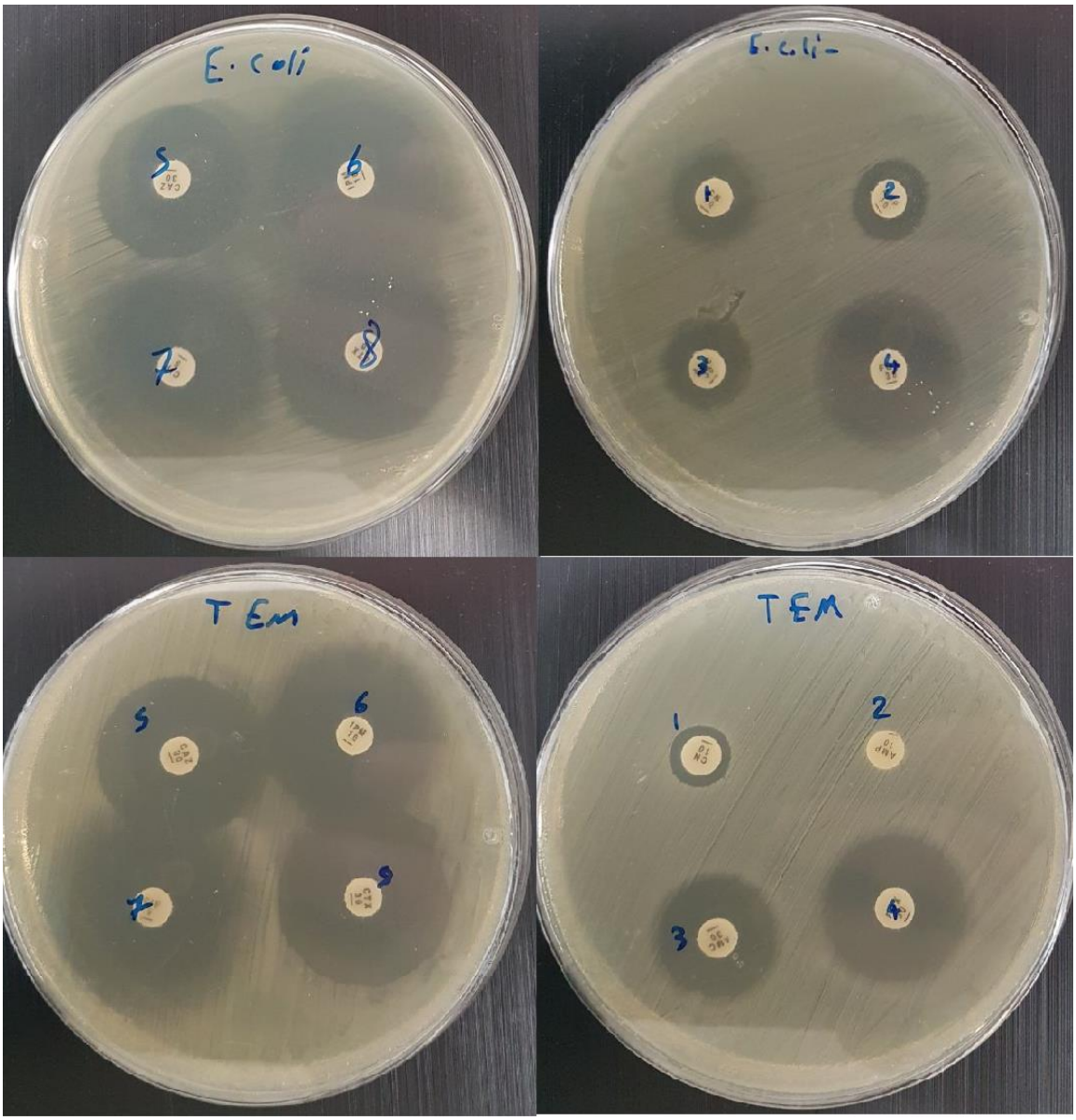

Figure 2.16: TEM strain showing resistance to Gentamicin and Ampicillin antibiotics compared with ESBL –ve strain which is resistant to Ampicillin antibiotic out of eight.

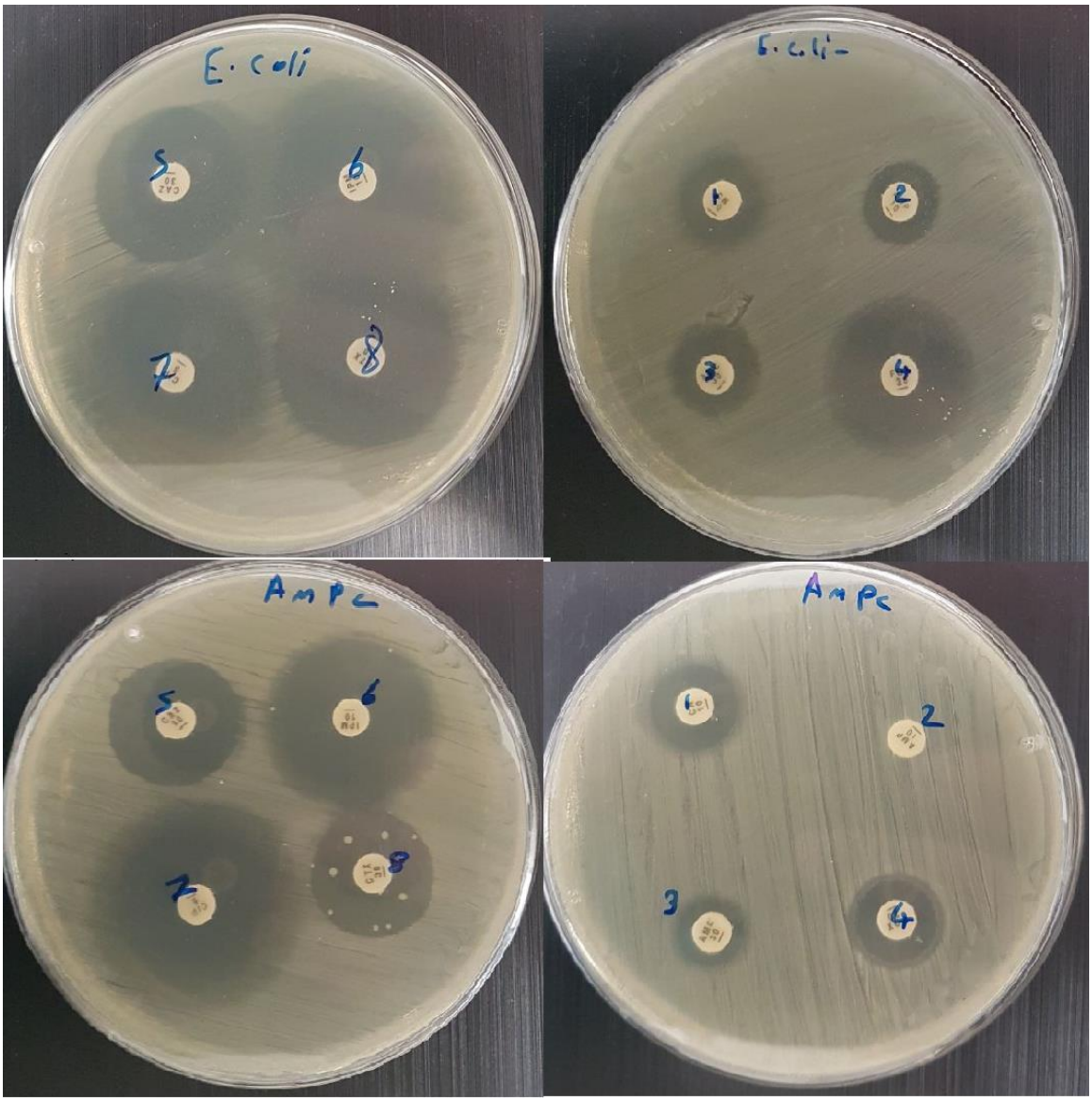

Figure 2.17: AmpC strain showing resistance to Ampicillin, Co-amoxiclave, and Cefoxitin antibiotics compared with ESBL –ve strain which is resistant to Ampicillin antibiotic out of eight.

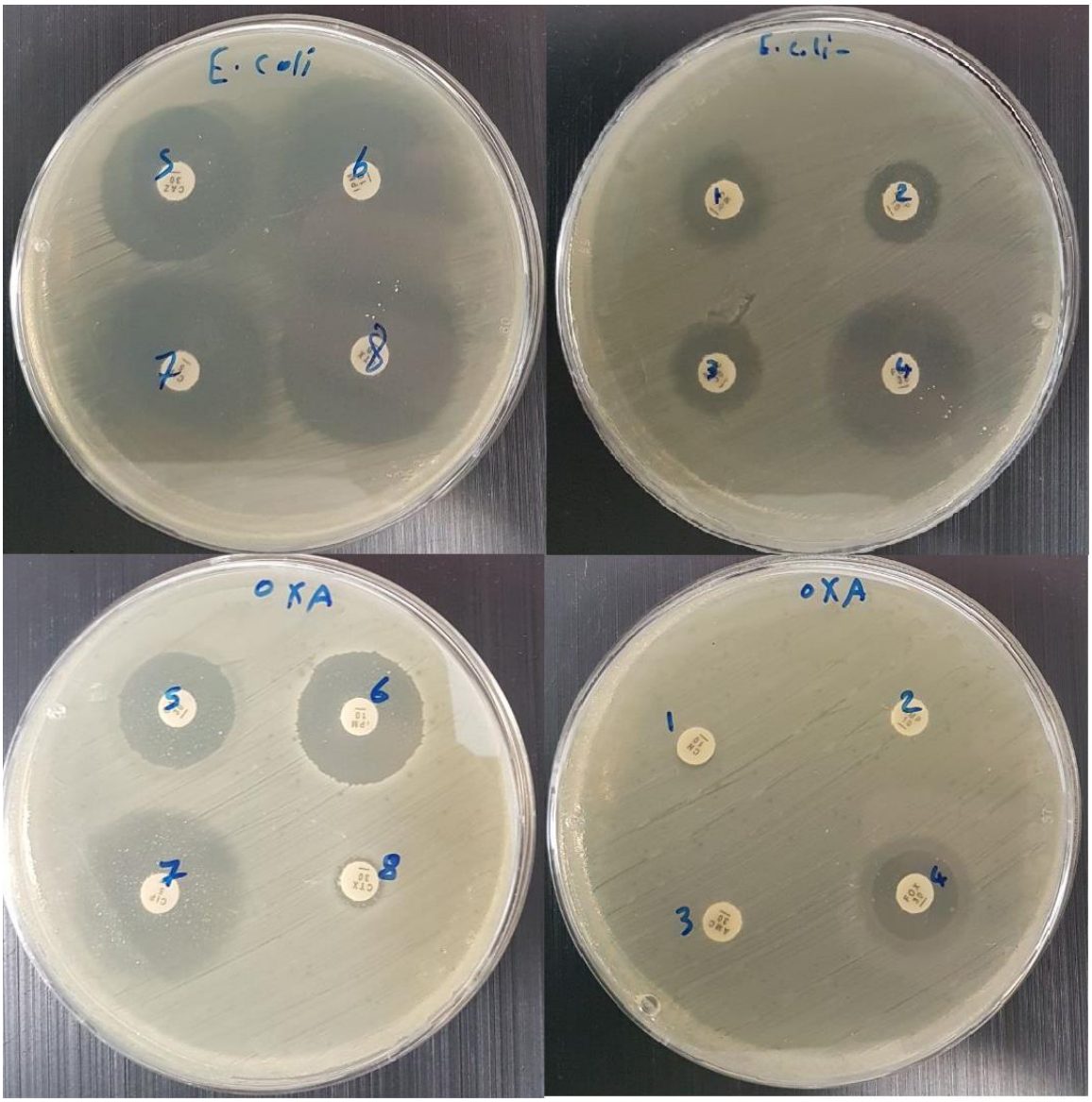

Figure 2.18: OX-48 strain is showing resistant to all antibiotics except Ciprofloxacin, and it showed intermediate sensitivity to Imipenem compared with ESBL –ve a strain which is resistant to Ampicillin antibiotic out of eight.

2.3.5 Susceptibility testing by minimum inhibitory concentration (MIC) (E- Test)

The antimicrobial sensitivity of each of the clinical strains was determined using the MIC of amoxicillin/clavulanic acid (Augmentin) results showed that AmpC and OXA-48 strains were resistant to Augmentin. As shown in Table 2.8, 128mg/l is the minimum concentration of antibiotic to inhibit the growth of AmpC and OXA strains. However, TEM strain was sensitive, with a MIC of 4mg/l, and the ESBL –ve *E. coli* strain was also sensitive with a MIC of 2mg/l, (Table 2.8 and Figure 2.19).

Table 2.8: Susceptibility testing of ESBL +ve and –ve strains of *E. coli* against amoxicillin/clavulanic acid antibiotic (MIC breakpoint (mg/L).

Antibiotics	**Code/ Concentration (µg)**	***E. coli* –ve ESBL**		***E. coli* +ve ESBL (TEM)**		***E. coli* +ve ESBL (AmpC)**		***E. coli* +ve ESBL (OXA)**	
		MIC		MIC		MIC		MIC	
Amoxycillin/ Clavulanic Acid	256µg/ml-0.015µg/ml	2	S	4	S	128	R	128	R

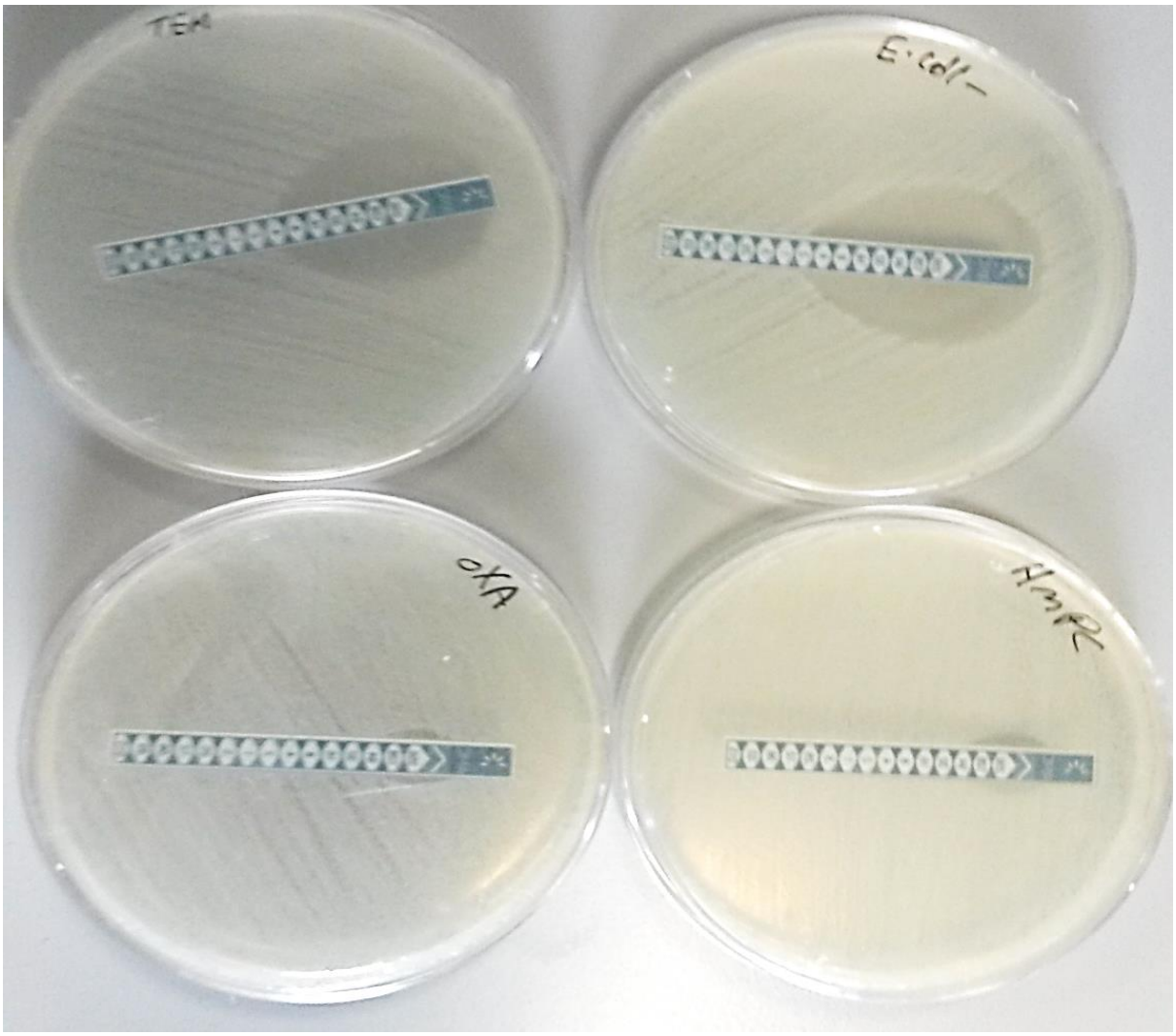

Figure 2.19: The MIC of Augmentin against ESBL +ve strains (TEM, AmpC, and OXA-48) compared with the control ESBL –ve strain.

2.4 Discussion

To confirm the identity of the bacteria used in this study, as well as to show the differences between them, molecular techniques such as PCR, protein profiles by SDS-PAGE and 2-D gel electrophoresis were used. The results were compared with a control ESBL -ve strain. Six sets of primers (*E. coli* 16S r*RNA*, TEM, AmpC, OXA-48, CTX-M and usp) were used to amplify the *E. coli* 16s rRNA, *TEM*, *AmpC* and *OXA-48* genes.

In this study, the PCR amplification of the *16S rRNA*, *TEM*, *AmpC* and *OXA-48* genes was carried out on the chromosomal DNA of the clinical isolates of *E. coli*; an additional PCR amplification of AmpC was carried out on plasmid DNA. These results of PCR were in concordance with previous studies (Table 2.1) for all primers used, confirming that these strains have unique characteristics and are different from one another. The OXA 48 isolate is known to produce the CTX-M and TEM β-lactamases (Cuzon *et al.*, 2011). Results from PCR confirmed that the OXA-48 strain used in this study bears both the *CTX-M* and *TEM* genes which are responsible for the production of CTX-M and TEM beta-lactamases. Also, the *usp* gene is considered an essential factor in infectivity (Yamamoto *et al.*, 2001). Recently *usp* has been described as a genotoxin with DNase activity that disrupts tight junctions and the actin cytoskeleton of host cells (Nipič *et al.*, 2013). While *usp* has been identified in 80-94% of urinary *E. coli*, this genotoxin appears to be absent from UPEC clones (Kurazono *et al.*, 2000; Johnson *et al.*, 2009; Skjøt-Rasmussen *et al.*, 2013). In this study, all strains of *E. coli -ve*, TEM and AmpC, except the OXA-48 strain, had *usp* gene, as described in Figure 2.5.

Proteomic research is a powerful study provides identification, structure and function of proteome expressed in a specific type of cells under specific environmental conditions. In addition, application of mass spectrometry (MS) in proteomics analysis has made it a potent tool for protein characterisation (Cravatt et al., 2007). In this study, SDS-PAGE was used to further

characterise the strains. The results of the protein profile were not decisive, and not showed significant differences between strains. Therefore, the 2-D gel was employed to obtain preliminary data about potentially different protein expression. The 2-D gel is considered one of the most powerful tools for protein separation and fractionation since its introduction in 1975 (Klose, 1975; O'Farrell, 1975; Reed *et al.*, 2012); indeed, this method has been proved the most robust tool for proteome analysis. Protein spots in the gels were stained with silver staining and analysed by the software Progenesis Same Spot, as previously used in a number of studies (Brasier *et al.*, 2012; Brioschi *et al.*, 2013). Results obtained from this analysis were used as preliminary data to identify differences among cellular and extracellular protein profiles of ESBL+ve and -ve *E. coli* strains.

The results for extracellular proteases by 2-D gel showed that many protein spots featured in both OXA-48 and AmpC strains, as compared with others. Some spots also featured in TEM strains, with many spots absent in ESBL -ve *E. coli* strain. The results of this proteomic analysis revealed the resolution of a number of polypeptide spots. Just one protein spot resolved at a given isoelectric point (pI) and the molecular weights were then identified. The protein search engine Uniprot used for protein identity analysis showed that the spot number 2656 was present in all ESBL strains but absent in the ESBL -ve *E. coli* strain, revealing potentially useful results. For example, the polypeptide spot number 2656 correlates with the potassium channel toxin alpha-KTx 9.10 protein, which has a pI of ~5.46 and an MW of ~30 kDa and is involved in blocks of shaker potassium channels. On the other hand, several other studies have also documented the role of potassium channel toxin alpha-KTx, which is perhaps the toxin of most uropathogenic *E. coli* strains. Alpha-KTx is expressed as a fusion protein with the mannose-binding protein in *E. coli* (Huys *et al.*, 2002; Nekrasova *et al.*, 2018). Further studies should be performed to sequence the protein spots to shed more light on the similarities/dissimilarities between the *E. coli* strains under investigation.

The intracellular protein results by 2-D gel indicated that many spots appeared on the OXA-48 strain, in contrast with others Table 2.6. Some spots also featured in AmpC and TEM strains; though many spots were absent in the ESBL -ve *E. coli* strains. The results of this proteomic analysis revealed the resolution of a number of polypeptide spots. Five protein spots resolved at a given pI, and molecular weights were identified. Uniprot was used to identify the proteins featured in all ESBL strains but absent in ESBL -ve *E. coli* strains. The polypeptide spot number 932 correlates with the probable Fe (2+)-trafficking protein (pI of ~5.71 and MW of ~110 kDa), which could be a mediator in iron transactions between iron acquisition and iron-requiring processes, such as synthesis and/or repair of Fe-S clusters in biosynthetic enzymes. Additionally, the spot number 1642 could be related to the phosphatidylserine decarboxylase proenzyme that catalyses the formation of phosphatidylethanolamine (PE) from phosphatidylserine at a pI of ~6.1 and has MW of ~60 kDa. Of note, the importance of PE metabolism in mammalian health has emerged following its association with the virulence of certain pathogenic organisms (Calzada *et al.*, 2016), and it is the second most abundant glycerophospholipid in eukaryotic cells. The spot number 1734 with a pI of ~5.25 and MW of ~55 kDa could be related to the putative uncharacterised protein DDB_G0284813. The spot number 2460 could be related to the conotoxin LeD51, which has a pI of ~6.15 and an MW of ~37 kDa and plays a role in toxin and ion channel inhibitor activity. The spot number 2747, with a pI of ~5.38 and an MW of ~32 kDa, could be related to the conotoxin flf14c, which has toxin activity.

Further mass spectrometry analysis is necessary to identify the sequence and identity of the differentially regulated polypeptide spots observed. This would validate the findings of this study and correlate with existing proteins identified in databases.

Molecular techniques are not usually utilised for the diagnosis of ESBL resistance in clinical practice. The benefits of such molecular techniques include testing methodologies to

identify the resistance determinant expressed when coupled with epidemiological resistance information, the most appropriate agent to possibly improving patient diagnosis, care and treatment.

The ESBLs are the most common groups of β-lactamases in clinical practice, as ESBL producers have the ability to develop resistance to penicillin, and to first-, second- and third-generation cephalosporins, but can be inhibited by β-lactamase inhibitors, such as a clavulanic acid (Rawat and Nair, 2010a). However, disc diffusion and MIC tests alone are not enough to identify ESBLs (Park *et al.*, 2014). It has been confirmed that the OXA-48 strain producer exhibits elevated MICs for broad-spectrum cephalosporins, such as cefotaxime and ceftazidime (Oueslati *et al.*, 2015). An earlier published reported that *E. coli* isolated from UTI infections were resistant to frontline antibiotics, with increased resistance to ciprofloxacin and trimethoprim (Croxall *et al.*, 2011). Similar levels of antibiotic resistance were observed in *E. coli* UTI isolates in this study, but higher levels of antimicrobial resistance were observed in the *E. coli* (OXA-48) strain.

The most common antibiotics that are used to treat UTIs were employed in this work, which include gentamicin, ampicillin, co-amoxycillin, ceftazidime, imipenem, ciprofloxacin, cefotaxime and cefoxitin. In these results of the antimicrobial sensitivity using the EUCAST method were in accordance with a previous study on the enzyme kinetics of OXA-48, which has a low level of hydrolytic activity against carbapenems, and better activity against imipenem than meropenem (Evans and Amyes, 2014). Our findings showed that the OXA-48 strain was resistant to all antibiotics, but sensitive to ciprofloxacin and intermediately so to imipenem. The MIC results indicated that the AmpC and OXA-48 strains were resistant to amoxicillin/clavulanic acid, with a MIC of 128 mg/l.

Resistance to ciprofloxacin by *E. coli* isolates has been reported in many studies, which concluded that these elevated levels of resistance were observed in community-acquired *E. coli* infections, due to drug misuse in outpatients and increased prescriptions selected for resistant mutants (Cooke *et al.*, 2010; Sahuquillo-Arce *et al.*, 2011). In fact, infection with an ESBL-producing *E. coli* isolate was one of the predictors of mortality due to community-acquired *E. coli* bacteraemia (Kang *et al.*, 2010; Bou-Antoun *et al.*, 2016). Further studies to sequence and analyse proteins on 2-D gel will shed more light on the identity and behaviour of multi-drug resistant *E. coli* strains.

CHAPTER THREE 3

3. Interaction of the uropathogenic bacteria with *Acanthamoeba* and urothelial cell line.

3.1 Introduction

Urinary Tract Infections (UTIs) usually start with periurethral infection, urethral colonisation, and then migration to the urinary bladder. In the bladder, host-pathogen interactions lead to successful colonisation or removal (Flores-Mireles *et al.*, 2015). Colonisation results in the formation of Intracellular Bacterial Communities (IBCs) (Rosen *et al.*, 2007; Flores-Mireles *et al.*, 2015). The cycle of IBC pathogenesis is characterised by uropathogenic association, invasion and survival within the urothelium (Rosen *et al.*, 2007). IBCs act to protect bacteria from the immune response and from antibiotic treatment, but also release intracellular bacteria into the surrounding environment to initiate new IBC cycles (Robino *et al.*, 2013). It has been suggested that the primary source of recurrent infections might be because of the re-emergence of bacteria from the intracellular communities and subsequent colonisation of new cells (Anderson *et al.*, 2004; Wright *et al.*, 2007).

Organisms which cause UTIs are microbes such as bacteria, viruses, and fungi. Bacteria are the most common kind of microorganisms to cause UTIs (Nicolle, 2001). Including many species of gram-negative Enterobacteriaceae, related to the lipopolysaccharide (LPS) layer of the gram-negative bacterial cell envelope (Madigan *et al.*, 2009). *E. coli* that cause UTIs are classified as uropathogenic *E. coli* (UPEC), and these strains account for 75–90% of uncomplicated UTIs, and can also cause bacteraemia, sepsis, and meningitis (Ahmed *et al.*, 2014). Briefly, the UTIs are the result of the interaction between the pathogens and the host. *E. coli* strains are highly adaptable, possessing a number of virulence factors which can enable the bacteria to colonise, infect the urinary tract, and then lead to UTIs.

ESBLs are enzymes produced by different gram-negative bacteria, especially by *E. coli* which can break down many types of antibiotics, leading to their inactivation. ESBLs have contributed importantly to the worldwide problem of antibiotic resistance since infections caused

by enzyme-producing organisms are linked with high morbidity and mortality rates and the financial impact of resistance can be a costly treatment (Davies and Davies, 2010; Salverda *et al.*, 2010; Winkler *et al.*, 2015). β-lactamases are hydrolytic enzymes which cleave the β-lactam ring conferring bacterial resistance to β-lactam antibiotics, such as penicillins and cephalosporins (Dhillon and Clark, 2012). In 2011, the European Centre for Disease Control and prevention (ECDC) stated that the spread of ESBL +ve *E. coli* had increased in more than half of the 28 European countries over the previous four years (Beytur *et al.*, 2015).

E. coli is considered the most general cause of UTIs, many studies having indicated that there are individual virulence factors (VF) which enhance the ability of *E. coli* to cause disease in a particular host (Moreno *et al.*, 2005; Wiles *et al.*, 2008). Actually, investigation of the VFs in certain strains is essential to understand the mechanism of their actions and also to develop specific anti-virulence factors to inhibit infection (Kawamura-Sato *et al.*, 2010).

E. coli adhesins can be broadly categorised as fimbrial like type P or afimbrial. These virulence factors give rise to the severe primary stage of infection: attachment to host cells or tissues. This binding helps the establishment of a bacterial community, in order to initiate infection. Type 1 fimbriae are the most common, typically identified in 88%-100% of *E. coli* strains, plus other members of the Enterobacteriaceae family, with a higher incidence in virulent strains (Blum *et al.*, 1991; Johnson, 1991; Moreno *et al.*, 2005; Cooke *et al.*, 2010). These fimbriae also help the establishment of intracellular reservoirs within bladder cells, which play a role as a source for recurrent UTI (Anderson *et al.*, 2004). On the other hand, P-fimbriae or pap (pyelonephritis-associated pili) is the second-most prevalent fimbrial type (Blum *et al.*, 1991; Narciso *et al.*, 2012; Norinder *et al.*, 2012). There are three forms of these fimbriae, with different binding affinities for the cells (uroepithelial and erythrocytes) and the host (human or animal) that they bind (Strömberg *et al.*, 1990). The Afa/Dr family of adhesins bind several epitopes of collagen and blood group antigens in the attachment process (Nowicki *et al.*, 1990;

Servin, 2005, 2014). The increased serum survival (Iss) protein has been detected on colV plasmids, with roles in serum resistance and surface exclusion (Johnson *et al.*, 2006; Mahdi *et al.*, 2015; Xu *et al.*, 2018).

The other microorganism of interest in this study is *Acanthamoeba*, is microscopic, which exists in two forms: trophozoites or cysts, found in soil, dust, and water. *Acanthamoeba spp.* Can cause severe infections such as keratitis, encephalitis, and Disseminated Infection (Dl). Previous studies showed that the pathogenic *E. coli* K1 strain interact with *Acanthamoeba* and can invade and remain viable inside *Acanthamoeba* while the non-invasive *E. coli* K-12 was killed (Alsam *et al.*, 2006). Trophozoites can actively replicate, feeds on bacteria, and cause infection in humans. In unfavourable conditions, for example, extreme temperature and pH, *Acanthamoeba* forms a double-walled cyst, which is the dormant, resistant stage of its life cycle (Khan, 2009; Siddiqui and Khan, 2012).

Acanthamoeba, in their trophozoite stage feed on bacteria, algae and fungi through phagocytosis, a contact-dependent mechanism (Alsam *et al.*, 2005; Khan, 2009). Conversely, the intracellular environment of *Acanthamoeba* can protect some bacterial endosymbionts from unfavourable conditions like the human immune response (Iovieno *et al.*, 2010). Based on Abd et al., (2003) studies have shown that the highly infectious has the ability to grow and survive intracellularly in *Acanthamoeba castellanii*. Also, many other studies have been conducted to confirm the endosymbiotic relationship between *Acanthamoeba* and bacteria. For example, *Vibrio cholerae* (Abd *et al.*, 2004), *E. coli* (Alsam *et al.*, 2006) and neuropathogenic *E. coli* (Yousuf *et al.*, 2014) have all been able to survive and multiply within *Acanthamoeba*.

Bacteria can develop different relationships with amoeba, and the nature of this relationship is determined by environmental conditions and virulence of the two organisms. According to Jeon (1995), there can be three outcomes of the relationship between amoeba and

bacteria, i.e. symbiosis, destruction of amoeba or destruction of bacteria. Researchers have found that certain bacterial species demonstrate greater virulence when grown with amoeba in comparison to the same bacterial species living in another environment. Some bacterial species which may develop a relationship with amoeba include *E. coli* (Alsam *et al.*, 2006), *Campylobacter jejuni* (Bui *et al.*, 2012), *Vibrio cholerae* (Abd *et al.*, 2009), and *Shigella sonnei* (Jeong *et al.*, 2007).

Researchers have found that bacteria can grow inside free-living *Acanthamoeba* as well as in human macrophages implying that these two types of cells share certain features (Cardas *et al.*, 2012). In these cases, phagosomes do not fuse with lysosomes. After 8h, the bacteria cause disruption of the membrane of the phagosome and enter the cytoplasm both in *Acanthamoeba* and macrophages (Abu Kwaik *et al.*, 1998). Intriguingly, the bacterial cells kept on proliferating inside *Acanthamoeba* as well as macrophages even after disruption of the phagosome and spreading of lysosomal constituents, cytoplasmic vesicles, mitochondria and amorphous entities (Molmeret *et al.*, 2004).

The urothelium lines the inner surface of the renal pelvis, ureters, bladder, and glandular ducts of the prostate. It forms a common boundary between the urinary space and the underlying tissues. The primary urothelial mediated response to infections is the exfoliation of superficial cells and urothelial regeneration. Also, it can promote dissemination of bacteria into deeper layers of the urothelium potentially benefiting both host and pathogen (Nagamatsu *et al.*, 2015).

Finally, interaction assays were performed to study the ability of different *E. coli* strains to interact with *Acanthamoeba* and TERT-NHUC cell line. PCR was conducted to investigate the virulence factors of ESBL+ve and -ve *E. coli* strains determinants adhesins and increased serum survival.

3.2 Materials and methods

All the chemicals for this study were bought from Sigma Laboratories and Thermofisher unless otherwise stated.

3.2.1 Bacterial culture

All strains were cultured onto CLED plates overnight, A few colonies were inoculated in LB broth, and ODs were measured by using spectrophotometer as explained in chapter 2 (2.2.1).

3.2.2 *Acanthamoeba* culture

For this study, *Acanthamoeba castellanii* belonging to the T4 genotype (ATCC 30234) was used. It is a clinical isolate from a keratitis patient, was kindly provided by Dr. S. Maciver, University of Edinburgh, UK. *Acanthamoeba* was cultured in 10ml PYG medium [proteose peptone 0.75% (w/v), yeast extract 0.75% (w/v) and glucose 1.5% (w/v)] in T-75 culture flasks at 30°C (Alsam *et al.*, 2006). Under these conditions, *Acanthamoeba* becomes confluent within two days (~$5x10^6$ cells per ml).

3.2.3 Cell line and cell culture

Normal Human Urothelial Cell Line (TRET-NHUC) was kindly provided by Prof. Knowles, from Cancer Research UK Clinical Centre, St. James's University Hospital in Leeds. The TRET-NHUC cell line was provided as a frozen sample. The frozen sample was in a mixture of growth medium with 10% Dimethyl sulfoxide (DMSO) and 10% fetal calf serum (FSC). That freezing medium was removed by centrifuging the thawed sample (1000 x g for 5min). The cells were re-suspended in 5ml Keratinocyte Growth Medium 2 (KGM2) with

supplements (purchased from Promo Cell). Finally, cells were passaged into T25 tissue culture flasks and incubated at 37°C in a CO_2 incubator (95% humidity, 5% CO_2). The medium was changed every two days. Permanently, cells become confluent within 4 days (~$1x10^6$ cells/ml). Then, the cells were ready to be seeded into 24-well plates or passaged into three new T25 flasks (Kavaler, 2007; Chapman *et al.*, 2008). All passages used in this study were between passage numbers 4 to 8.

3.2.4 Artificial Urine Medium (AUM)

The Artificial Urine Medium (AUM) was prepared according to the analysis of human urine constituents. All components in Table 3.1 were well mixed, and the pH was adjusted to 6.5. Then, the medium was sterilised by passing through a 0.2µm nylon membrane filter under vacuum suction. After that, the AUM was divided into 50ml tubes and freeze-stored (Brooks and Keevil, 1997). To determine the effects of AUM and AUM without urea on bacteria (*E. coli* (ESBL –ve), TEM, AmpC, and OXA-48) were used.

Table 3.1: AUM composition(Brooks and Keevil, 1997)**.**

Component	**Quantity (g)**
Peptone L37	1
Yeast extract	0.005
Lactic acid	0.1
Citric acid	0.4
Sodium bicarbonate	2.1
Urea	10
Uric acid	0.07
Creatinine	0.8
Calcium chloride ($2H_20$)	0.37
Sodium chloride	5.2
Iron II sulphate ($7H_20$)	0.0012
Magnesium sulphate ($7H_20$)	0.49
Sodium sulphate ($10H_20$)	3.2
Potassium dihydrogen phosphate	0.95
Di-potassium hydrogen phosphate	1.2
Ammonium chloride	1.3
Distilled water	To 1L

3.2.5 Interaction assays

The percentage of bacteria interacted with *Acanthamoeba* and TERT-NHUC line was calculated as follows:

The number of viable bacteria recovered (CFU/ml) /a number of bacteria in initial

inoculum x 100 (Alsam *et al.*, 2006; Yousuf *et al.*, 2013).

3.2.5.1 Association assays

To determine the effect of urine on the interaction of bacteria with *Acanthamoeba* and TERT-NHUC line, AUM was used as the growth medium, and association assays were performed (Figure 3.1). Briefly, *Acanthamoeba* or TERT-NHUC line were cultured and grown in 24-well plates in their own medium [PYG for *Acanthamoeba* and KGM2 for cell line] (~$5x10^6$ cells per ml). Plates were incubated until confluency. Bacterial strains (~1×10^6 CFU/ml and ~1×10^7 CFU/ml) were added onto *Acanthamoeba* and TERT-NHUC line in 24-well plates with medium and incubated for one hour at RT. Supernatants were discarded, and wells were washed with PBS once to remove non-adherent bacteria. *Acanthamoeba* or TERT-NHUC line were lysed by adding 150µl Cell Lytic M for 2min. Finally, 20µl of the lysed solution was cultured onto CLED agar and incubated overnight at 37ºC. The bacteria associated with *Acanthamoeba* or TERT-NHUC line were calculated as % bacteria associated with cells= no. of bacteria recovered / original number of bacteria in each well × 100 (Alsam *et al.*, 2006).

3.2.5.2 Invasion assays

Invasion assays were performed to determine the ability of bacteria to invade *Acanthamoeba* and TERT-NHUC line (Figure 3.1). In brief, *Acanthamoeba* or TERT-NHUC line were cultured and grown in 24-well plates in own medium as mentioned in 3.2.5.1. Plates were incubated until confluency. Bacterial strains at 10 and 100 bacteria/cell (~1×10^6 CFU/ml and ~1×10^7 CFU/ml) were added onto *Acanthamoeba* or TERT-NHUC line with AUM medium and incubated for 1h at RT. After that, wells were washed with PBS, and then antibiotics were added to kill extracellular bacteria (meropenem with final concentration of 150µg/ml against *E.coli* (ESBL-ve, TEM, and AmpC) meropenem with a final concentration of 200µg/ml against *E.coli* (OXA-48) for 60min). Finally, wells were washed with PBS, and then cells were lysed by adding 150µl Cell Lytic M for 2min, and 20µl of the lysed solution was cultured onto CLED

agar, incubated overnight in the 37^{o}C incubator. The intracellular bacteria were calculated as follows: the number of bacteria invaded each cell = no. of bacteria recovered/no. of cells in each well × 100 = percentage of intracellular bacteria (Alsam *et al.*, 2006).

3.2.5.3 Intracellular survival assays

Intracellular survival assays were performed to determine the reproduction and growth of bacteria inside *Acanthamoeba* or TERT-NHUC line (Figure 3.1). Briefly, *Acanthamoeba* or TERT-NHUC line were incubated with bacteria, followed by the addition of antibiotic (meropenem) for 60min, as described above in invasion assays section. After incubation, wells were washed with PBS. *Acanthamoeba* or TERT-NHUC lines were incubated in 0.5ml AUM medium for 24h at 30^{o}C. Finally, *Acanthamoeba* or TERT-NHUC line were lysed as mentioned earlier. Then, 20ul of the lysates were cultured onto CLED plates, and these were incubated overnight at 37^{o}C. The survival of intracellular bacteria after 24h incubations was calculated as described in invasion assays section (Alsam *et al.*, 2006).

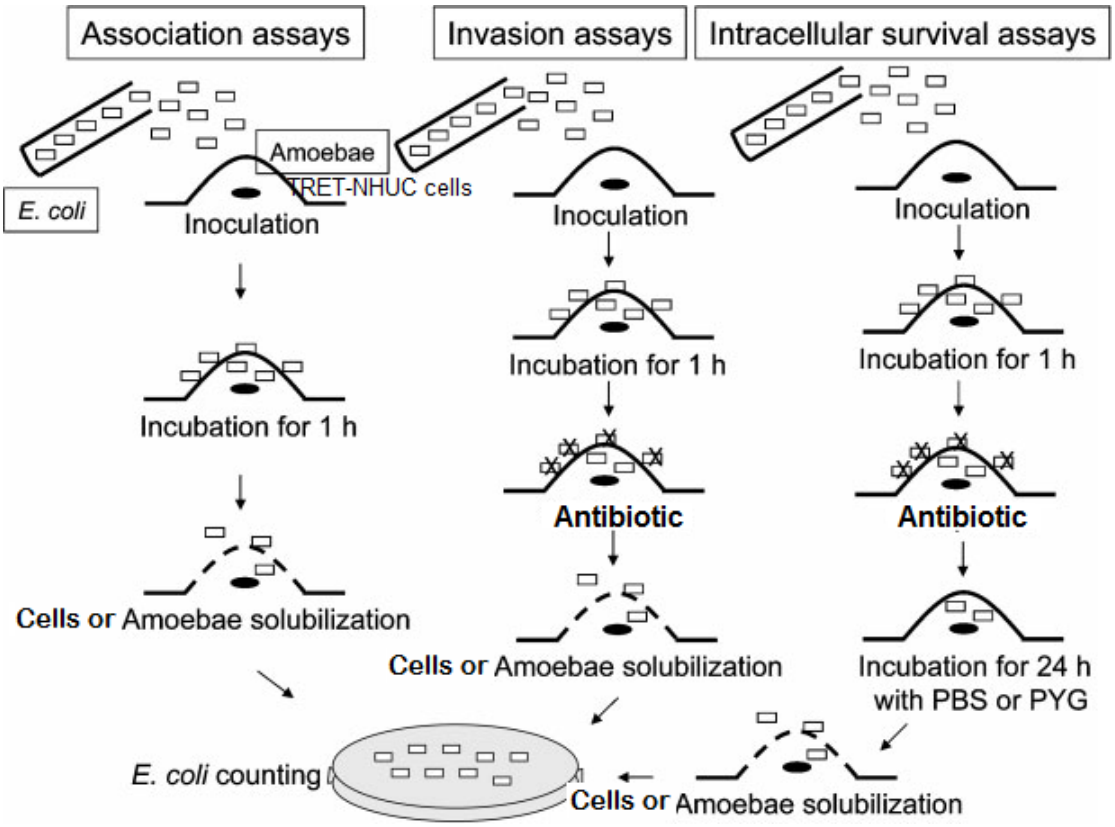

Figure 3.1 Steps of bacterial interaction assays (association, invasion and survival) with *Acanthamoeba* (Alsam *et al.*, 2006).

3.2.5.4 Statistical analysis

Microsoft Office Excel programme (v15.30) was used for general data analysis to prepare graphs and to calculate the means, standard deviation and standard error. To find the significant differences between bacterial strains, a one-tailed t-test was used to calculate P-value unless otherwise stated.

3.2.6 Polymerase chain reaction

3.2.6.1 DNA extraction

Bacterial DNA was extracted according to the manufacturer's instructions to perform PCR as explained in chapter 2 (2.2.2).

3.2.6.2 Polymerase Chain Reaction

In order to characterise and identify virulence determinants involved in adhesion and Increased serum survival in the study strains, the presence or absence of multiple genes were probed. PCR amplification using specific primers (PapA-F, PapC-F, FimH, Afa/draBC, and iss) were performed (Table 3.2). Each one of the PCR reactions was carried out in 50 µl reaction volumes in the PCR tube as described in chapter 2 (2.2.5.1). The PCR cycling setup was different for each gene as listed in (Table 3.3).

Table 3.2 Gene and primer sequences for the virulence factors of *E. coli*.

Target gene	Primer (nucleotide sequence) Sequence (5`-3`)	reference	Product length (bp)
PapA	F- ATGGCAGTGGTGTCTTTTGGTG	(Johnson and Stell, 2000)	717
	R- CGTCCCACCATACGTGCTCTTC		
PapC	F- GTGGCAGTATGAGTAATGACCGTTA	(Johnson and Stell, 2000)	205
	R- ATATCCTTTCTGCAGGGATGCAATA		
FimH	F- TCGAGAACGGATAAGCCGTGG	(Johnson and Stell, (2000)	508
	R- GCAGTCACCTGCCCTCCGGTA		
Afa/deaBC	F- GGCAGAGGGCCGGCAACAGGC	(Johnson and Stell, 2000)	559
	R- CCCGTAACGCGCCAGCATCTC		
Iss	F- CAGCAACCCGAACCACTTGATG	(Johnson and Stell, 2000)	323
	R- AGCATTGCCAGAGCGGCAGAA		

Table 3.3 The setup of PCR cycling for the different genes under investigation.

PapA, PapC, FimH, and Afa	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	12	00
Denaturation	94 ºC	00	30
Annealing	63 ºC	00	30
Extension	68 ºC	03	00
Final Extension	72 ºC	10	00
Cycles	25		
iss	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	15	00
Denaturation	94 ºC	00	30
Annealing	68 ºC	00	30
Extension	72 ºC	01	00
Final Extension	72 ºC	05	00
Cycles	30		

3.3 Results

3.3.1 Association assays of *Acanthamoeba* T4

Association assays were performed to determine the ability of bacteria to associate with *Acanthamoeba* T4 in AUM. All bacterial strains were incubated in a 24- well microtiter plate seeded with *Acanthamoeba* for one h at 30ºC.

As shown in Figure 3.2, all strains displayed significant ($p<0.05$) ability to associate with *Acanthamoeba*. The most striking result is that ten bacteria per amoeba associated with *Acanthamoeba* more than 100 bacteria per amoeba. Our findings showed that *E. coli* (AmpC) strain exhibited the least association with *Acanthamoeba* when using 10 or 100 bacteria/ amoeba (4.43% and 3.53% bacteria per amoeba). The most significant in these results, the association of OXA-48 with *Acanthamoeba* was much higher than other pathogenic lines. OXA-48 showed the highest association with *Acanthamoeba* for 10 bacteria/amoeba (8.77% bacteria per amoeba) and 100 bacteria/amoeba (6.63% bacteria per amoeba) as described in (Figure 3.2). Overall, OXA-48 ESBL+ve strain displayed the highest ability to associate with *Acanthamoeba* T4 this was higher than the control as well.

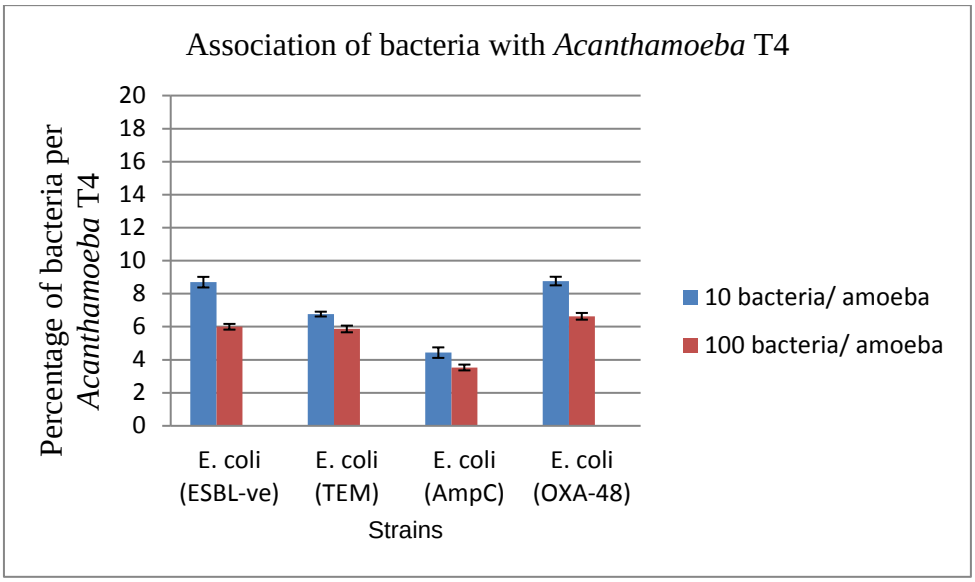

Figure 3.2 The percentage of bacteria associated per *Acanthamoeba* T4. Results are the mean of three autonomous experiments carried out in duplicate. Error bars represent standard error. Statistical analysis: one-tailed t-test (P<0.05). The association of OXA-48 with *Acanthamoeba* was much higher than other pathogenic lines when using 10 or 100 bacteria/ amoeba. However, *E. coli* (AmpC) strain exhibited the least association with *Acanthamoeba* when using 10 or 100 bacteria/ amoeba.

3.3.2 Association assays of TRET-NHUC line

Association assays were performed to determine the ability of bacteria to associate with TRET-NHUC line in AUM. All bacterial strains were incubated in a 24-well microtiter plate seeded with urothelial cells for 1h at 37°C in humidified 95% air and 5% CO_2.

As shown in Figure 3.3, all strains displayed significant ($p<0.05$) ability to associate with urothelial cells. The most significant result that is ten bacteria per cell associated with urothelial cells more than 100 bacteria per cell. *E. coli* (AmpC) strain exhibited the least significant association with urothelial cells when using 10 or 100 bacteria/cell (6.33% and 3.5% bacteria per cell). Association of *E. coli-ve* for 10 bacteria per cell with urothelial cells was significant much higher than other strains it was 19.33% bacteria per cell (Figure 3.3).

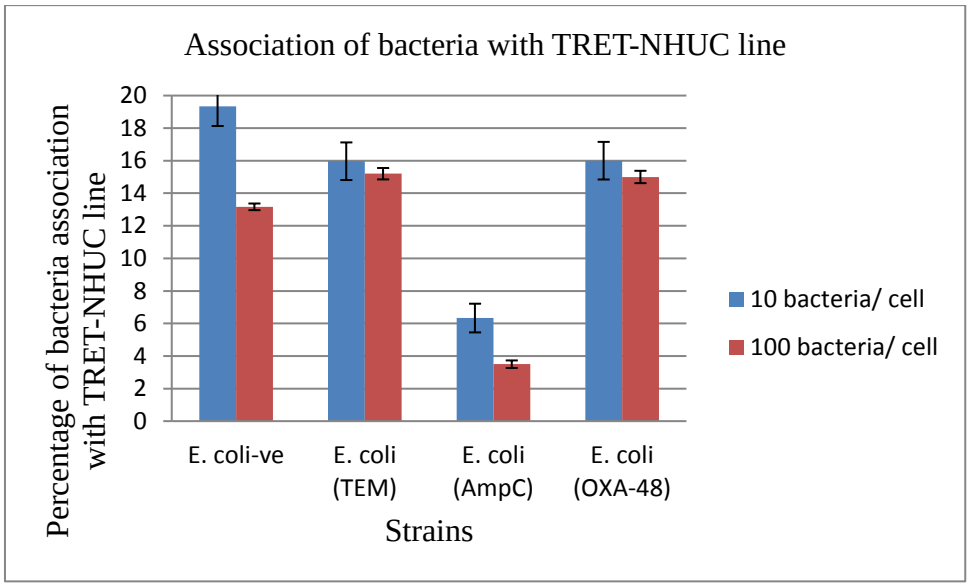

Figure 3.3 The percentage of bacteria associated per TRET-NHUC line. Results are the mean of three autonomous experiments carried out in duplicate. Error bars represent standard error. Statistical analysis: one-tailed t-test (P<0.05). *E. coli* (AmpC) strain exhibited the least significant association with urothelial cells when using 10 or 100 bacteria/cell.

3.3.3 Invasion assays of *Acanthamoeba castellanii*

To define whether bacteria were devoured by *Acanthamoeba* T4, invasion assays were carried out. All bacterial strains were recovered from *Acanthamoeba*. Uropathogens associated with *Acanthamoeba* T4 were treated with antibiotics to kill extracellular bacteria. The results obtained from the invasion assays are shown in Figure 3.4. It is apparent from this figure that all bacterial strains were able to invade *Acanthamoeba* albeit at different levels ($p<0.05$ in comparison with the initial inoculum).

These results have been shown; ten bacteria per amoeba invade *Acanthamoeba* more than 100 bacteria per amoeba. The highest level of invasion with *Acanthamoeba* was with OXA-48 10 bacteria/amoeba (0.337% bacteria per amoeba) ($P<0.05$ using paired t-test, one-tailed distribution). TEM strain displayed less invasion compared with the other strains for 10 or 100

bacteria/amoeba (0.18% and 0.061% bacteria/amoeba). AmpC strain 10 or 100 bacteria/amoeba displayed less invasion compared with the higher strain it was (0.21% and 0.116% bacteria/amoeba).

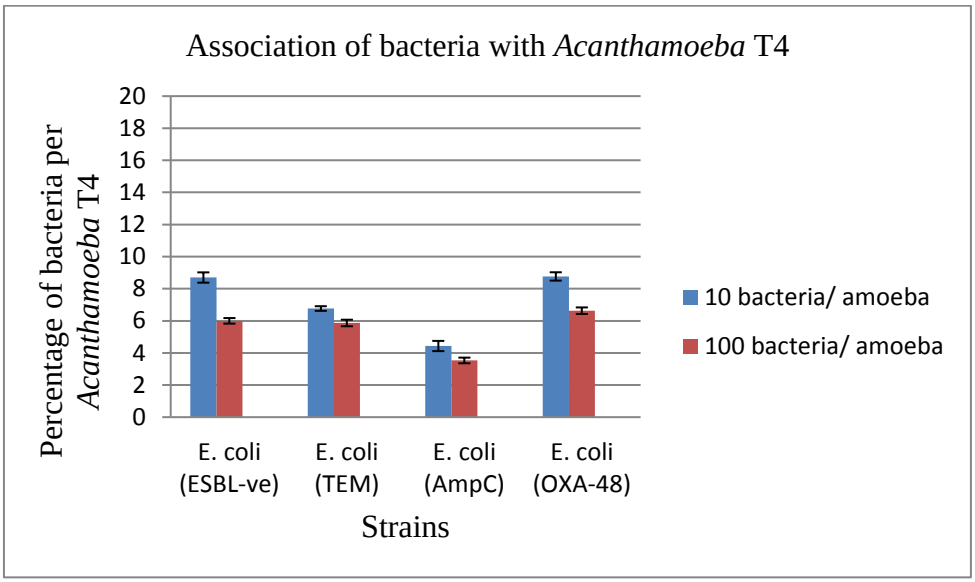

Figure 3.4 The percentage of invaded bacteria per *Acanthamoeba* T4. Results are the mean of three autonomous experiments carried out in duplicate. Error bars represent standard error. Statistical analysis: one-tailed t-test (P<0.05). Ten bacteria per amoeba invade *Acanthamoeba* more than 100 bacteria per amoeba. The highest level of invasion with *Acanthamoeba* was with OXA-48 10 bacteria/amoeba.

3.3.4 Invasion assays of TRET-NHUC line

To determine the ability of uropathogens to invade TERT-NHUC line, invasion assays were performed. All bacterial strains were recovered from TERT-NHUC line. Uropathogens associated with TERT-NHUC line were treated with antibiotics to kill extracellular bacteria. The results obtained from the invasion assays are shown in Figure 3.5. It is apparent from this figure that all bacterial strains were able to invade TERT-NHUC line albeit at different levels ($p<0.05$ in comparison with the initial inoculum).

These results have been shown; 10 bacteria per cell invade with TERT-NHUC line more than 100 bacteria per cell. The highest level of invasion with TERT-NHUC line was with OXA-48 10 bacteria/cell (0.013% bacteria per cell) ($P<0.05$ using paired t-test, one-tailed distribution). AmpC strain displayed less invasion compared with the other strains for 10 or 100 bacteria/cell (0.009% and 0.05% bacteria/ cell). *E. coli-ve* and TEM strains with 10 bacteria/cell displayed similar invasion with urothelial cells; there were 0.01% 10 bacteria/cell. However, TEM strain with 100 bacteria/cell (0.009% bacteria/cell) displayed higher than *E. coli-ve* strain with 100 bacteria/cell (0.05% bacteria/cell).

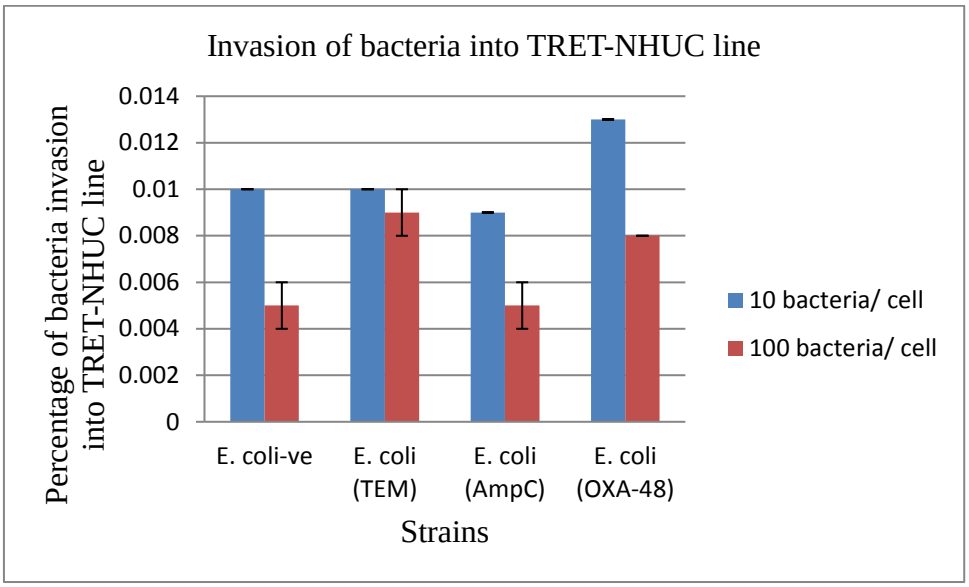

Figure 3.5 The percentage of invaded bacteria per TRET-NHUC line. Results are the mean of three autonomous experiments carried out in duplicate. Error bars represent standard error. Statistical analysis: one-tailed t-test ($P<0.05$). Ten bacteria per cell invade with TERT-NHUC line more than 100 bacteria per cell. The highest level of invasion with TERT-NHUC line was with OXA-48 10 bacteria/cell.

3.3.5 Intracellular survival assays of *Acanthamoeba castellanii*

Survival assays were carried out to determine the fate of bacteria inside *Acanthamoeba* by incubating with internalised bacteria for 24h. *Acanthamoeba* T4 invaded with bacteria were

incubated at 30°C for 24h. Data obtained (Figure 3.6) from the survival assays reveal that not all uropathogens were able to survive within *Acanthamoeba*.

Our results showed that OXA-48 was the only strain which survived and multiplied inside the *Acanthamoeba*. OXA-48 at 10 bacteria/amoeba had the highest survival inside the *Acanthamoeba* (13.5% bacteria per amoeba) than OXA-48 on 100 bacteria/amoeba (6.767% bacteria/amoeba) ($P<0.05$ using paired t-test, one-tailed distribution).

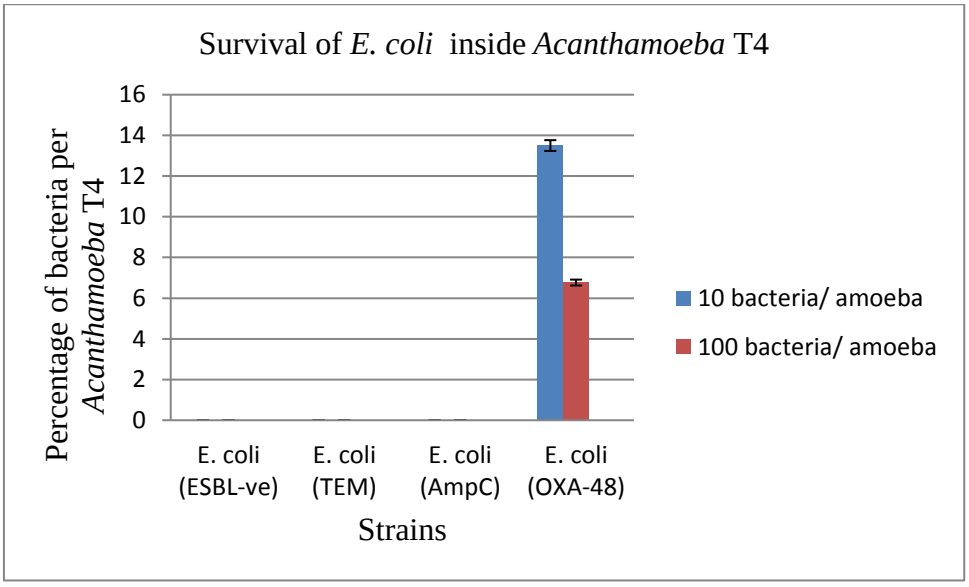

Figure 3.6 The percentage of bacteria survive inside *Acanthamoeba* T4. Results are the mean of three autonomous experiments carried out in duplicate. Error bars represent standard error. Statistical analysis: one-tailed t-test ($P<0.05$). OXA-48 was the only strain which survived and multiplied inside the *Acanthamoeba*. OXA-48 at 10 bacteria/amoeba had the highest survival inside the *Acanthamoeba* than OXA-48 on 100 bacteria/amoeba.

3.3.6 Intracellular bacterial survival assays in TRET-NHUC line

Survival assays were carried out to determine the fate of bacteria inside TRET-NHUC line by incubating with internalised bacteria for 24h. Urothelial cells invaded by bacteria were incubated at 37°C in humidified 95% air and 5% CO_2 for 24h. Data obtained (Figure 3.7) from the survival assays reveal that not all uropathogens were able to survive within urothelial cells.

The significant in these results showed that OXA-48 was the only strain which survived and multiplied inside the cell line. OXA-48 at 10 bacteria/cell had the highest survival inside the cell line (0.017% bacteria per cell) compared to OXA-48 at 100 bacteria/cell (0.013% bacteria/cell). ($P<0.05$ using a paired t-test, one-tailed distribution).

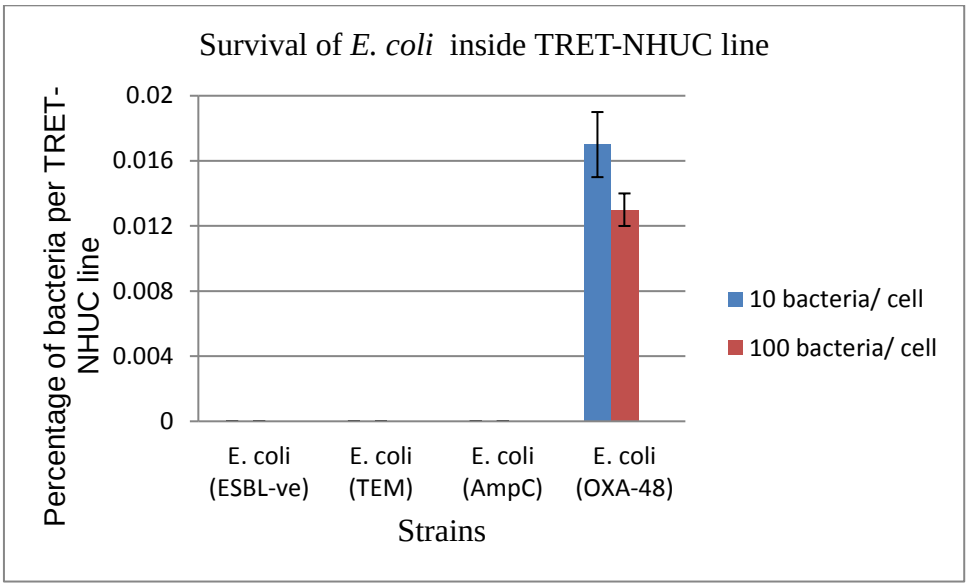

Figure 3.7 The percentage of bacteria surviving inside TRET-NHUC line. Results are the mean of three autonomous experiments carried out in duplicate. Error bars represent standard error. Statistical analysis: one-tailed t-test ($P<0.05$). OXA-48 was the only strain which survived and multiplied inside the cell line. OXA-48 at 10 bacteria/cell had the highest survival inside the cell line compared to OXA-48 at 100 bacteria/cell.

3.3.9 Polymerase Chain Reaction (PCR)

Results obtained from this study showed a variation in the virulence factors borne by the ESBL strains under study (Table 3.4). The ESBL strains were investigated for *E. coli* (VFs), using an updated version (personal correspondence from Prof. James) of Johnson and Stell's PCR assays (Johnson and Stell, 2000; Johnson *et al.*, 2013). Five sets of primers (PapA, PapC, FimH, Afa/deaBC, and Iss) were used to study the VFs amplified from the genomic DNA of the strains under study (ESBL +ve (TEM, AmpC, and OXA-48) and ESBL–ve). PCR amplification

was carried out on the chromosomal DNA of the clinical strains of *E. coli*. The PCR cycling setup was different for each gene as listed in (Table 3.3).

All the strains under study are seen to bear the *FimH* gene. The most important of these results is that just the OXA-48 strain was seen to have *Iss* gene; this gene was absent from the other strains under study. The PCR products from the FimH Primer to ESBL –ve *E. coli*, TEM, AmpC, and OXA-48 were observed, and corresponded to the expected results, with approximately 508 bp being successfully amplified. The PCR products from the PapA primer amplified by just ESBL–ve *E. coli* was observed as a band, corresponding to the expected results, with approximately 717 bp successfully amplified, as described in (Figure 3.8). On the other hand, for TEM, AmpC, and OXA-48 no band was observed. The PCR products when using the PapC primer were amplified for the ESBL –ve *E. coli* and TEM strains with correspondence to the expected result, with approximately 205 bp successfully amplified, as described in (Figure 3.8). The PCR products when using the Afa/deaBC primer were amplified. For the ESBL –ve *E. coli* and AmpC *s*trains, no band was observed. On the other hand, just a band from TEM and OXA-48 corresponded to the expected result, with approximately 559 bp successfully amplified, as described in (Figure 3.8).

Table 3.4: Summary of virulence factors genes amplified from the genomic DNA of the strains under study (ESBL-ve and ESBL +ve (TEM, AmpC, and OXA-48).

Strains	PapA	PapC	FimH	Afa/ deaBC	Iss
E. coli-VE	+	+	+	-	-
TEM	-	+	+	+	-
AmpC	-	-	+	-	-
OXA-48	-	-	+	+	+

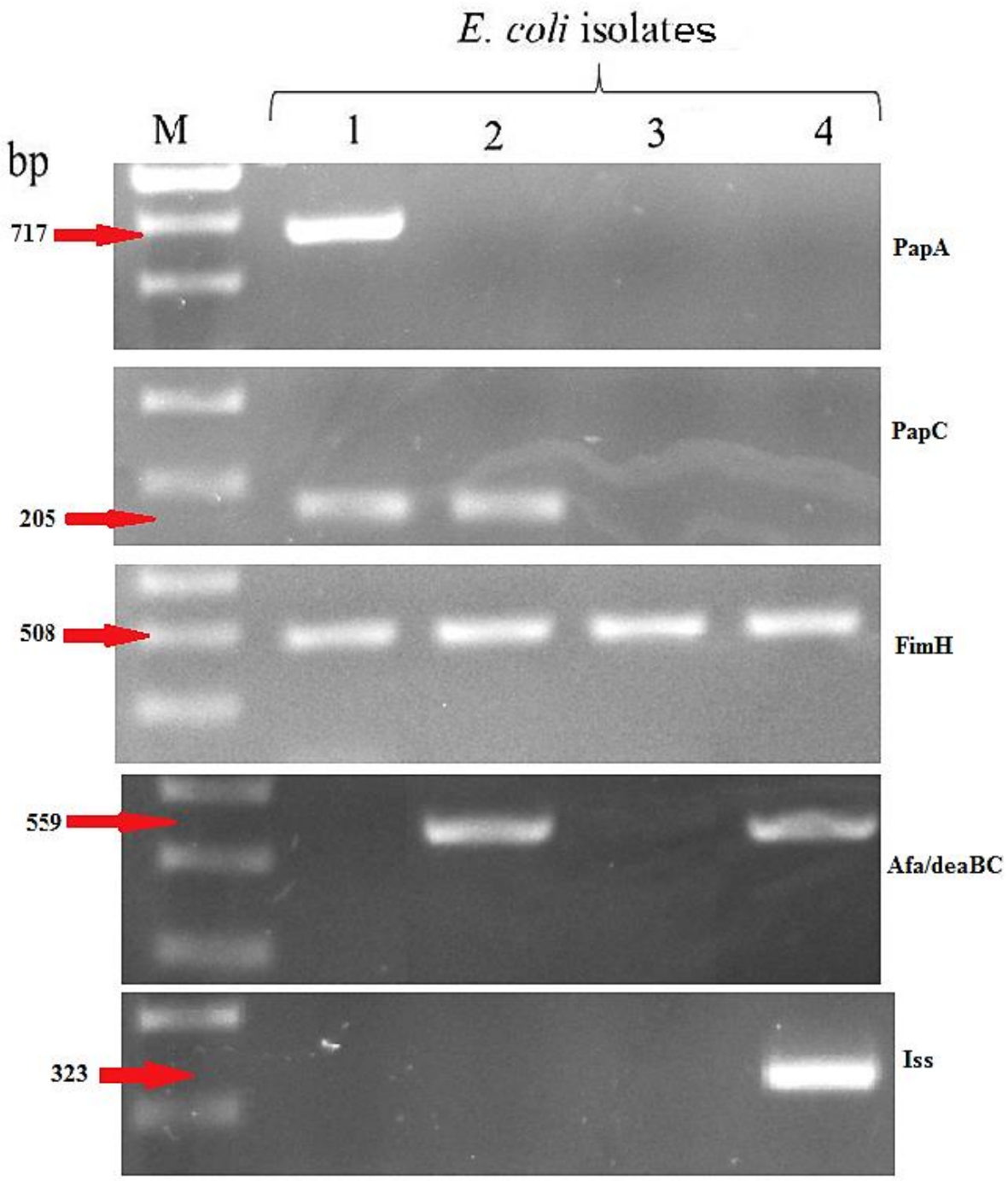

Figure 3.8: PCR products amplified using PapA, PapC, FimH, Afa/deaBC, and Iss primers. Bands were observed in all lanes corresponding to the expected (PapA (717bp), PapC (205bp), FimH (508bp), Afa/deaBC (559bp), and Iss (323bp). M: Marker. Lanes 1- *E. coli* ESBL –ve, 2- TEM, 3- AmpC and 4- OXA-48.

3.4 Discussion

This study set out with the objective of exploring the inter-relationship between uropathogens and *Acanthamoeba* T4, and the interrelationship between uropathogens and the TERT-NHUC cell line.

It is scientifically known that *Acanthamoeba* is pathogenic to humans, mainly causing AGE and AK. Previous studies have shown the presence of *Acanthamoeba* in ocular clinical samples (Spanakos *et al.*, 2006; Ozkoc *et al.*, 2008; Cave *et al.*, 2014). Also, *Acanthamoeba* has been found in brain tissue (Yagi *et al.*, 2007), cerebrospinal fluid samples (Sheng *et al.*, 2009), sputum samples (Bradbury *et al.*, 2014). Also, of great interest in the present context, in 2009, Santos et al. found in 26% of urine samples under investigation *Acanthamoeba* spp in urine samples.

A fundamental question is how bacteria interact with urothelial cells of the urinary tract causing infections. Furthermore, the presence of *Acanthamoeba* with the urothelial cells could contribute to the exacerbation of urinary tract infections. For this reason, in this study, *Acanthamoeba* T4 trophozoites, human urothelial cells (TRET-NHUC line), and four pathogenic *E. coli* types of bacteria (ESBL -ve, TEM, AmpC, and OXA-48) isolated from UTI patients were studied. To identify if bacteria interact with *Acanthamoeba* or urothelial cells, interaction assays were carried out. Proteases produced by *Acanthamoeba* are used as significant markers to differentiate between pathogenic and non-pathogenic *Acanthamoeba* species (Khan *et al.*, 2000; Sissons *et al.*, 2006; Dudley *et al.*, 2008). *Acanthamoeba* proteases have antimicrobial effects against different types of bacteria (Iqbal *et al.*, 2014). Our findings indicate that *Acanthamoeba* (genotype T4), was able to host *E. coli* (OXA-48). Moreover, bacteria can remain and multiply inside *Acanthamoeba* within a harsh environment, where they can turn to cysts that may help transmit bacterial pathogens to susceptible hosts and also to cause reinfection. The results clearly

showed that all bacteria used have the ability to associate and invade with *Acanthamoeba* T4 and host cells. However, only OXA-48 was able to survive within *Acanthamoeba* T4 and urothelial cells.

Acanthamoeba acts as a host and trojan horse for several bacteria especially pathogenic strains that can live and multiply inside (Cardas *et al.*, 2012; Cardas, 2014). The relationship for the interactions between *Acanthamoeba* and bacteria are complex, diversified and depend on the number and species of bacteria. However, the mechanisms associated with bacteria–amoebae interactions are still incompletely understood (Alsam *et al.*, 2006; Siddiqui and Khan, 2012). It is possible to lead to amoebic destruction, bacterial destruction or the development of a symbiotic relationship (Marciano-Cabral and Cabral, 2003). Alsam et al. (2006) proved that *E. coli* interactions with *Acanthamoeba* depend on the virulence of the bacteria. To assess the nature of this relationship, interaction assays were performed. The current findings indicate that all uropathogens tested here are able to associate with and invade *Acanthamoeba* T4 albeit to different degrees. However, just OXA-48 was able to survive within *Acanthamoeba* T4.

The most surprising result, which is ten bacteria per amoeba associated with *Acanthamoeba* more than 100 bacteria per amoeba. Our findings showed that the OXA-48 strain had exhibited a higher capability for associating with *Acanthamoeba* T4 compared with the other uropathogens under study (Figure 3.2). A similar trend can be seen in the invasion assays (Figure 3.4), both when using 10 or 100 bacteria/*Acanthamoeba*. Moreover, OXA-48 seems to exceed the other uropathogens in their ability to survive and multiply with amoebae (Figure 3.6). According to Khan (2009), several bacteria including *E. coli* were more resistant to free chlorine in the presence of *Acanthamoeba* suggesting an increase in virulence. Also, many studies have reported similar findings as mentioned in the literature review (Chapter one). From this study, the results of interaction assays support the hypothesis that uropathogens possibly use

Acanthamoeba to survive under severe conditions and cause recurrent infections in the urinary tract.

As discussed above, the IBC cycle consists of association, invasion, and survival. IBC pathogenic cycle has been investigated *in vitro* studies but has not yet been investigated in humans (Rosen *et al.*, 2007). In a previous study conducted on urine samples collected from women with cystitis, Rosen et al., (2007) have found evidence of IBCs and filamentous bacteria in 18% and 41% of urine samples respectively, suggesting evidence of an IBC pathway in humans. Also, many studies have documented the interaction of bacteria with urothelial cells (Garofalo *et al.*, 2007; Rosen *et al.*, 2007; Berry *et al.*, 2009; Robino *et al.*, 2013).

Results from the current study (Figure 3.3), indicate that all uropathogens under study are able to interact with TERT-NHUC line with ESBL negative strain of *E. coli*, displaying higher levels of interaction than ESBL+ve strains of *E. coli*. Our findings showed that *E. coli-ve* and OXA-48 strains exhibited a higher capability for associating with urothelial cells compared with the other uropathogens under study (Figure 3.3). However, the OXA-48 strain with 100 bacteria per cell has a higher capability for associating with urothelial cells than *E. coli-ve* and the rest of the uropathogens under study. For the invasion assays also OXA-48 when using 10 bacteria/cell has a higher ability to invading onto urothelial cells compared with the other uropathogens under study (Figure 3.5). Moreover, OXA-48 when using 10 or 100 bacteria/cell seems to exceed the other uropathogens in their ability to survive and multiply with urothelial cells (Figure 3.7).

These findings showed that the highest level of association with *Acanthamoeba* and TRET-NHUC line was with *E. coli-ve* and OXA-48 with both infection dose (10 and 100 bacteria/amoeba or cell line). However, it was higher with infection dose 10 bacteria/amoeba or cell line than 100 bacteria/amoeba or cell line. In invasion assays, it was found that *Acanthamoeba* had a higher bacterial strains recovery rate than TRET-NHUC line. In addition,

results indicated that *E. coli-ve*, TEM, and AmpC were not able to survive and multiply in *Acanthamoeba* as well as TRET-NHUC line while OXA-48 had a higher level of survival with *Acanthamoeba* than TRET-NHUC line. It is evident from these results that OXA-48 strain has the highest level of association with *Acanthamoeba* and TRET-NHUC line, and this is consistent with previous findings where OXA-48 was found to be more virulent and resistant than the other strains. Previous studies (mostly with uropathogenic *E. coli*) have documented the interaction of bacteria with NHUC, thus, contributing to the pathogenesis of urinary tract infection (Bower *et al.*, 2005; T. J. Wiles *et al.*, 2008; Bang *et al.*, 2014).

In the present study, several virulence genes (for adhesins and increased serum survival) were compared between four clinical strains of *E. coli* (ESBL +ve and ESBL–ve). Results obtained from this study showed a variation in the VFs borne by the ESBL strains under study (Table 3.4). The ESBL strains were investigated for *E. coli* (VFs) by using five sets of primers (PapA, PapC, FimH, Afa/deaBC, and Iss) (Johnson and Stell, 2000; Valat *et al.*, 2012; Raeispour and Ranjbar, 2018). PCR amplification was carried out on the chromosomal DNA of the clinical strains of *E. coli*. Each PCR reaction was carried out in 50µl reaction volumes in the PCR tube. The PCR cycling setup was different for each gene as listed in (Table 3.3). Then, the size of the fragments was observed under a UV light.

Pathogenic *E. coli* strains, cause different infections, including (UTI). One of the reasons these strains cause diseases is because they have many VFs that may play a role in infection by enabling the bacterial cells to move into the host and disseminate. *E. coli* VFs include adhesion molecules and toxins (Mora-Rillo, Fernández-Romero, Francisco, *et al.*, 2015). Adherence to the host cells can be done by fimbriae or pili, however; they are complex structures and thus encoded by gene clusters coding for instance by fimbrial subunits, assembly and secretion machinery. These fimbriae contain a rod, composed hundreds to thousands of different major subunits, and the adhesive tip, formed by single or few minor subunits. Inside the periplasmic

space, the chaperon facilitates folding of the subunits, which are then assembled and secreted in the outer membrane (Waksman and Hultgren, 2009).

P-fimbriae or pap (pyelonephritis-associated pili) is the second-most prevalent fimbrial type causing pyelonephritis in UPEC (Blum *et al.*, 1991; Narciso *et al.*, 2012; Norinder *et al.*, 2012). There are three forms for these fimbriae, with different binding affinities affecting the cells (uroepithelial and erythrocytes) and the host (human or animal) that they bind (Stromberg et al. 1990). Type 1 fimbriae are a critical VFs in Uropathogenic *E. coli* (UPEC), although these pili are not restricted to pathogenic strains (Vejborg *et al.*, 2011; Norinder *et al.*, 2012). Type 1 fimbriae are essential to colonise the urinary tract in humans (Bahrani-Mougeot *et al.*, 2002), however, alone they are not enough to establish a long-term infection (Mulvey *et al.*, 2001). FimH is one of these type 1. The adhesive tip of these fimbriae, FimH, binds to α-D-mannosylated proteins like UPs, which are expressed by the differentiated urothelium.

E. coli strains that carry coding for Afa/Dr adhesins are referred to as diffusely adherent. Usually, this VF is associated with intestinal infections especially in young children, but also it is found among strains from extra-intestinal infections including UTI (Le Bouguénec and Servin, 2006). So far, the complete receptor repertoire of all Afa/Dr adhesin subtypes has not been fully identified, but some of it may influence the association of certain strains to different tissues. For instance, Afa/Dr subtype afaE8 has been found highly concentrated between UPEC from pyelonephritis (Le Bouguénec *et al.*, 2001).

The primary function of Iss protein is to increased serum survival. (Iss) Protein plays roles in serum resistance and surface exclusion (Binns *et al.*, 1982; Waters and Crosa, 1991; Pitout, 2012). Iss is less diffuse in strains causing bacteraemia (23% vs 68%), UTI (17% vs 65%) (Waters and Crosa, 1991; Johnson *et al.*, 2005; Olesen *et al.*, 2012; Skjøt-Rasmussen *et al.*, 2013). The most important in these results, just the OXA-48 strain was seen to have the *Iss* gene,

and this gene was absent from the other strains under study. The PCR products from the *FimH* gene were seen to occur in all the strains under investigation, the *PapC* gene was seen on ESBL –ve *E. coli* and TEM strains (Figure 3.8). The *PapA* gene was just demonstrated in ESBL –ve *E. coli* strain. On the other hand, the *Afa/deaBC* gene was observed on TEM and OXA-48 strains. The higher rate of survival and multiplication observed in the OXA-48 strain can also be attributed to horizontal gene transfer which always occurs between *E. coli* strains (Juhas, 2015). This is because OXA-48 may have acquired virulence genes which enhance its survival and multiplication. For instance, the *Iss* gene, a plasmid (colV) bore gene which is responsible for survival in serum, was found in the OXA-48 but not in the other strains (Figure 3.8). The presence of this gene may be the reason why the OXA-48 strain survived and multiplied better than others (sections 3.3.5 and 3.3.6). The observed differences in survival and multiplication could also be a function of strain variability, i.e. differences in the genome-encoded virulence determinants of the different strains.

In conclusion, the findings from this chapter primarily suggest that OXA-48 strain can survive and multiply with *Acanthamoeba* (T4). Therefore, it is reasonable to hypothesise that *Acanthamoeba* may play a crucial role in causing rUTIs. By using the PCR method in the present study, it was demonstrated that just OXA-48 strain has the *Iss* gene and that only OXA-48 can survive in *Acanthamoeba* and TRET-NHUC line. The dissemination of bacteria from their intracellular reservoirs is a clinically important stage of the IBC cycle, because of their relevance to recurrent UTIs (Robino *et al.*, 2013). The dissemination stage of the IBC cycle can play an essential role in initiating new IBC cycles through colonisation of new cells and thereby causing recurrent UTIs. Nevertheless, the results from this chapter should provide the basis for further studies, particularly *in vivo*, to clarify the exact mechanism of host-pathogen interaction specifically for OXA-48. The next chapter aims to explore the cytotoxic effect of uropathogens and *Acanthamoeba* (T4) on NHUC.

Chapter four 4

4. Determination of cytotoxicity and Programmed Cell Death of uropathogens and *Acanthamoeba* on the urothelial cell line.

4.1 **Introduction**

Once attachment to host cells has been initiated, *E. coli* secrete toxins which damage host cells, facilitate invasion or destroy host tissues, so completing the second and last step of infection. Uropathogenic *E. coli* (UPEC) are involved in the great majority of UTIs. Most intracellular bacteria have the ability to stimulate host cell cytotoxicity by secreting different virulence factors (Lu *et al.*, 2013). Cellular cytotoxicity can lead to cell death by apoptosis and/or necrosis. Apoptosis is a programmed form of cell death used to eliminate cells that are not required and to maintain normal cell populations. Moreover, it can happen under both physiological and pathological conditions. Necrosis, by contrast, is cell death, which is non-apoptotic in nature. It is pathological and a pathway of cell death related to cell injury (Kumar *et al.*, 2015).

In apoptosis, cell death can happen by the intrinsic or extrinsic pathways also known as signalling cascades. The intrinsic or mitochondrial pathway is caused via the imbalance of B-cell lymphoma-2 (Bcl-2) family proteins causing increased mitochondrial permeability, leakage and caspase activation. The extrinsic or death receptor pathway is initiated by the engagement of plasma membrane death receptors on cells leading to the formation of 'death-inducing signalling complex' and finally caspase activation. On the other hand, in necrosis, the mechanism of cell death is associated with its specific pathology. In addition, the release of immunomodulatory factors, a significant immune response and phagocytosis are also characteristic of necrosis (Vanlangenakker *et al.*, 2008; Guicciardi *et al.*, 2013; Kumar *et al.*, 2015). Moreover, apoptosis and necrosis differ morphologically. The former is characterised by shrinkage of cells, nuclear changes such as pyknosis (nuclear shrinkage), and karyorrhexis (nuclear fragmentation). The characteristics of necrosis are swelling of cells, chromatin condensation, disrupted plasma membrane, leakage of cellular contents, and frequent adjacent inflammation (Kumar *et al.*, 2015). Apoptosis and necrosis morphological markers are essential to determine the stages of apoptotic

cell death. It is known that apoptosis and necrosis can coexist in the same tissue or cell type (Lu *et al.*, 2013). Early apoptotic cells are characterised by an intact cell membrane with cell surface expression of phosphatidylserine (PS) which promotes recognition by phagocytes. Early apoptotic cells become late apoptotic cells when the membrane becomes permeabilised, which can then progress on to secondary necrotic cells. Necrotic cells are characterised by membrane permeability; this is because of direct exposure of viable cells to trauma like chemical insults rather than a transition from apoptosis (Poon *et al.*, 2010).

Usually, Flow cytometry is used to differentiate dying cells. It is the favourite method for single-cell analysis of multiple cellular attributes (Wlodkowic *et al.*, 2009). Flow cytometry measures many characteristics of apoptotic cells like changes in the cell membrane, membrane permeability (cell and mitochondrial), caspase activation and DNA damage. It helps to quantify cells based on the stages of apoptotic cell death (Macey, 2007). Many studies have employed the use of Annexin V/Propidium Iodide for assessment of cell death. In early apoptotic cells, annexin V (Ca_2+ dependent phospholipid-binding protein, which has a high affinity for PS) binds to PS expressed on the outer leaflet of cell plasma membranes. However, in late apoptosis and necrosis, membranes of dead and damaged cells are permeable to propidium iodide (DNA stain). Flow- cytometric detection of apoptosis and necrosis depends on this differential staining. An alternative from flow cytometry for investigating cellular cytotoxicity with regards to the necrotic pathway is a measurement of lactate dehydrogenase (LDH). LDH is a soluble cytoplasmic enzyme released into the extracellular space through damaged cell membranes. The formazan product can be colourimetrically measured to give an estimate of the percentage of necrotic cells in a sample (Chan *et al.*, 2013).

The pathogenicity of *Acanthamoeba* infections is a complex process that includes greater adhesion properties, secreted cytotoxic products, invasion mechanisms and survival mechanisms (Arnalich-Montiel *et al.*, 2014). Although the secretions are not fully characterised, several

studies have highlighted their role in host pathology. Other studies have also investigated their antimicrobial potential (Alfieri *et al.*, 2000; Khan *et al.*, 2000; Harrison *et al.*, 2010). The pathogenesis of *Acanthamoeba* can be divided into two classes of mechanisms: contact-dependent and contact-independent mechanisms (Alsam *et al.*, 2003).

The most virulent of the contact independent factors of *Acanthamoeba* are extracellular proteases which lead to the death of the host cells (Garate *et al.*, 2005). The proteases are a group of enzymes which break down the peptide bonds on fibrinogen, albumin, haemoglobin, chemokines and cytokines. There are different types of proteases present in *Acanthamoebae* such as serine proteases, Metallo-proteases, and cysteine proteases (Dudley *et al.*, 2008). Also, there are different factors contributing directly to *Acanthamoebae* pathogenicity like Ecto-ATPases, Neuroaminidases, Superoxide dismutases, Elastases, and Glucosidases. Pathogenic strains of *Acanthamoeba* produce increased levels of protease activity and bring about higher levels of cytotoxicity than non–pathogenic strains. One of the proteases recognised was a 107 kDa serine protease (Khan *et al.*, 2000; Alsam *et al.*, 2005).

Most strains of *E. coli* cause UTIs and bacteraemia, neonatal meningitis, intra-abdominal infections, and others. Also, they have been characterised by the presence of specific virulence factors such as P-fimbriae, type 1 fimbriae, haemolysin, secreted autotransporter toxin (sat), serine protease (Pic), type II capsule, serum resistance proteins and cytotoxic necrotizing factor 1 (CNF1) (Johnson and Stell, 2000; Russo and Johnson, 2000; Smith *et al.*, 2007; Venier *et al.*, 2007; Johnson *et al.*, 2012).

Haemolysin A (HlyA) lyses erythrocytes allowing the producing strain to obtain iron for growth and survival in the absence of siderophores (Beutin, 1991; Mitsumori *et al.*, 1999; Norinder *et al.*, 2012). HlyA is perhaps the principal toxin of most *E. coli* strains, identified in

29%-52% of strains (Johnson and Stell, 2000; Moreno *et al.*, 2005; Cooke *et al.*, 2010; Mahjoub-Messai *et al.*, 2011).

The function of CNF1 is generating multinucleated cells that develop into necrotic lesions (Caprioli *et al.*, 1983; Johnson, 1991). CNF1 has been detected in 15%-54% of human *E. coli* strains causing UTI and bacteraemia (Johnson *et al.*, 2005; Venier *et al.*, 2007; Cooke *et al.*, 2010; Mahjoub-Messai *et al.*, 2011).

Secreted autotransporter toxin (Sat) is another most common *E. coli* toxin, which had been identified in 39%-94% of investigated strains (Pitout *et al.*, 2005; Johnson *et al.*, 2008; Mahjoub-Messai *et al.*, 2011). Although this toxin has rarely been screened for, likely due to its recent discovery (Guyer *et al.*, 2000). This toxin interferes with the cytoskeleton of epithelial cells and acts as a serine protease (Guyer *et al.*, 2000; Dobrindt *et al.*, 2002).

Pic plays a role in lysing mucin as part of diarrheal disease and mediates serum resistance (Henderson *et al.*, 1999; Olesen *et al.*, 2012). Moreover, Pic has also been identified in some strains of *E. coli* causing cystitis and pyelonephritis (Heimer *et al.*, 2004).

E. coli produces more than 80 different capsule types, or K antigens, which consist of acidic polysaccharides. The essential functions of the capsules are to facilitate the invasion of tissues and evasion of the host immune system (Huang *et al.*, 1999; Ananias and Yano, 2008). Previously, based on thermostability during serotyping, biochemical and genetic characteristics of *E. coli* capsules were categorised into three groups (1-3). However, there is a new capsule classification which includes four capsular groups (1-4) composed of O-antigens and/or K antigens. Not all strains of *E. coli* express K antigens, but many of the strains are capsulated, supporting the idea of capsules as an essential virulence factor (Orskov and Orskov, 1992; Whitfield and Roberts, 1999).

Finally, work in this chapter elucidates the cytotoxic effect of intracellular bacteria, and *Acanthamoeba* conditioned media by using flow cytometry and LDH assays to differentiate between apoptotic and necrotic cytotoxicity. Moreover, PCR was conducted to investigate the virulence factors of ESBL+ve and –ve *E. coli* strains determining their toxins and uropathogenic specific proteins.

4.2 Materials and methods

All the chemicals for this study were bought from Sigma Laboratories and Thermofisher unless otherwise stated.

4.2.1 Cell line and cell culture

Normal human urothelial cell line (TRET-NHUC) was grown and cultured as mentioned in section (3.2.3).

4.2.2 Bacterial culture

All strains were cultured onto CLED plates overnight, A few colonies were inoculated in LB broth, and ODs were measured by using spectrophotometer as explained in chapter 2 (2.2.1).

4.2.2.1 Heat-killed bacteria

Heat-killed bacteria were prepared to check whether the bacterial body has a role in cell death. All strains were refreshed onto CLED plates and incubated overnight. Next day, a few colonies were inoculated in LB broth and incubated overnight at 37^{o}C incubator. The OD was measured using spectrophotometer as described in section (2.2.1). Then, 1mL of bacterial broth (containing 10^{8} CFU) was heated in a water bath at 65^{o}C for 90 min. About 20µL of heat-killed bacteria were cultured onto CLED plates and incubated overnight at 37^{o}C in the air to be sure no bacteria were still alive, and no bacterial growth was detected after incubation (Worgall *et al.*, 2002).

4.2.2.2 Bacterial Conditioned Media (BCM)

BCM were prepared to explore the ability of proteases, other enzymes, and toxins which were produced by *E. coli* strains used in this study to have any role in cytotoxicity on the TERT-NHUC line. Bacterial strains (10^6 and 10^7 CFU) were inoculated in the 5ml of cell media of (Keratinocyte Growth Medium 2) in tubes. The tubes were then incubated at 37^{o}C overnight. After incubation, tubes were centrifuged at 5000 x g for 10min, and the supernatants were filtered twice by using syringe and filter pore size (0.20μm) to get conditioned media which contained the proteins and external virulence factors from all strains. Usually, BCM were stored at -20^{o}C no longer than two weeks (Iqbal *et al.*, 2014).

4.2.3 *Acanthamoeba* culture

Acanthamoeba T4 strain was grown and cultured as described in section (3.2.2).

4.2.3.1 *Acanthamoeba* Conditioned Medium (ACM)

Acanthamoeba T4 was used to study the cytotoxic role of it is ACM on the TERT-NHUC line. The culture medium from a confluent T75 flask of *Acanthamoeba* T4 (~5 x 10^6 *amoeba*/ml), obtained as described in section 3.2.2, was discarded. Afterwards, fresh KGM2 medium was added to the flask, and the cells were then incubated overnight at 30^{o}C. After that the medium was collected in 15ml tubes and centrifuged at 3000 x g for 5min; the supernatant was then filtered twice using 0.2μM filters to get conditioned medium which contained the proteins and external virulence factors of *Acanthamoeba* T4. Usually, ACM was stored at -20^{o}C no longer than two weeks (Dudley *et al.*, 2008; Iqbal *et al.*, 2014).

4.2.4 Programmed Cell Death (PCD) assays by flow cytometry

To study the ability of living, heat-killed and BCM of ESBL-ve and ESBL+ve bacterial strains used in this study, and CM of *Acanthamoeba* to induce cell death on TRET-NHUC cell line, flow cytometry assays were performed. TRET-NHUC were cultured and grown in 24-well plates in KGM 2 (~$5x10^5$ cells per well) in a CO_2 incubator (95% humidity, 5% CO_2) at 37^{o}C until 100% confluence was attained. The growth medium was then discarded following which cells were washed with PBS to remove non-adherent cells. KGM 2 and the required concentrations of the bacterial strains were added to all wells as required. Live or heat-killed bacteria were used at concentrations of 10/cell and 100/cell, while the BCM as prepared in section 4.2.2.2 was used. Control wells had KGM 2 added. Plates were subsequently incubated at 37^{o}C for 24h in a CO_2 incubator (95% humidity, 5% CO_2). CMA, as prepared in section 4.2.3.1, was used (10µl and 100µl volumes). Plates were subsequently incubated overnight at 37^{o}C in a CO_2 incubator (95% humidity, 5% CO_2). Afterwards, cells were harvested by adding 100µl Accutase (a cell detachment solution containing proteolytic and collagenolytic enzymes, it is an alternative for Trypsin-EDTA solution) and collected with the supernatants in Eppendorf tubes and centrifuged at 1000 x g for 5min. BioLegend's FITC Annexin V Apoptosis Detection Kit with Zombie NIR was used as per the manufacturer's instructions. Briefly, the cells were washed twice with 200µl of cold cell staining buffer and centrifuged each time at 1000 x g for 5min before being resuspended in 100µl of Annexin V binding buffer containing 5µl of Annexin V FITC and 1µl of Zombie NIR. Samples were gently vortexed and incubated for 20min at RT in the dark. Then, samples were washed with cell staining buffer, centrifuged at 1000 x g for 5min, and fixed with 450µl paraformaldehyde for 20min on ice. After fixation, the samples were centrifuged at 1000 x g for 5min, supernatants discarded and the pellets were washed once with cell staining buffer at 1000 x g for 5min. Then, the pellets were resuspended in 400µl of Annexin V binding buffer. Next, cells were analysed by using a flow cytometer [BD AccuriTM C6] with

each run restricted to 10000 moments, due to slow cell growth and that a large number of cell are needed. (Worgall *et al.*, 2002).

4.2.5 Cytotoxicity assays by lactate dehydrogenase (LDH)

These assays were performed to determine the cytotoxic effect of the bacterial strains used in this study and the ACM on TRET-NHUC cell line. TRET-NHUC were cultured and grown in 24-well plates in KGM2 (~$5x10^5$ cells per well) and incubated in a CO_2 incubator (95% humidity, 5% CO_2) at 37ºC until 100% confluence was attained. The growth medium was then discarded following which cells were washed with PBS to remove non-adherent cells. KGM2 and the required concentrations of the bacterial strains were added to all wells. Live bacteria were used to infect cells at the rate of 10 and 100 bacteria/cell. On the other hand, heat-killed bacteria were generated by heating bacteria in a water bath at 65ºC for 90min while cell-free bacterial condition media were prepared as described in section 4.2.2.2. Only KGM2 was added to designated high and low control wells. Plates were subsequently incubated for 24h at 37ºC in a CO_2 incubator (95% humidity, 5% CO_2). CMA, as prepared in section 4.2.3.1, was used (10µl and 100µl volumes). Only KGM2 was added to the designated high and low control wells. Plates were subsequently incubated for 24h at 37ºC in a CO_2 incubator (95% humidity, 5% CO_2). Following this incubation, the cytotoxic effects were determined by measuring the amount of lactate dehydrogenase -LDH (an enzyme released by dead cells) in the supernatants (Cytotoxicity Detection Kitplus; Roche). Lysis buffer (solution 3) was added to high control wells in order to bring about the release of a high amount of LDH from the cells while the low controls were just cells in growth medium. Fifty µl of the 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 15min at RT. Next, stop solution (solution 4) was added 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:

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

4.2.6 Software and statistics

FlowJo software (v10.1) was used to analyse and prepare flow cytometry graphs. In addition, Microsoft Office Excel programme (v15.30) was used for general data analysis to make graphs and to calculate the means, standard deviation and standard errors. To find the significant differences between bacterial strains, a one-tailed t-test was used to calculate P-value.

4.2.7 Genomic analysis

4.2.7.1 DNA extraction

Bacterial DNA for use in PCRs was extracted from all the bacterial strains according to the manufacturer's instructions as described in chapter 2 (2.2.2).

4.2.7.2 Identification of virulence determinants (toxins and capsule) by PCR

In order to characterise and identify virulence determinants, primers were used in PCR to probe the presence or absence of multiple genes. PCR amplification using specific primers (hlyD, CNF-1, pic, sat, and kbsII (Kii)) were performed (Table 4.1). Each one of the PCR reactions was carried out in 50 µl reaction volumes in the PCR tube as described in chapter 2 (2.2.5.1). The PCR cycling setup was different for each gene as listed in (Table 4.2).

Table 4.1 Gene and primer sequences for the virulence factors of *E. coli*.

Target gene	Primer (nucleotide sequence) Sequence (5`-3`)	reference	Product length (bp)
HlyD	F- CTCCGGTACGTGAAAAGGAC	(Johnson and Stell, 2000)	904
	R- GCCCTGATTACTGAAGCCTG		
CNF-1	F- GGGGGAAGTACAGAAGAATTA	(Tóth *et al.*, 2003)	1112
	R- TTGCCGTCCACTCTCACCAGT		
Sat	F- GCAGCTACCGCAATAGGAGGT	(Johnson and Stell, 2000)	937
	R- CATTCAGAGTACCGGGGCCTA		
pic	F- GGGTATTGTCCGTTCCGAT	(Wallace-Gadsden *et al.*, 2007)	1200
	R- ACAACGATACCGTCTCCCG		
Kbsll	F- GCGCATTTGCTGATACTGTTG	(Johnson and Stell, 2000)	570
	R- AGGTAGTTCAGACTCACACCT		

Table 4.2 The setup of PCR cycling for the different genes under investigation.

hlyD, pic, and CNF-1	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	15	00
Denaturation	94 ºC	00	30
Annealing	68 ºC	00	30
Extension	72 ºC	01	00
Final Extension	72 ºC	05	00
Cycles	30		
Sat and KbsII	Temperature	Time	
		min	s
Initial Denaturation	95 ºC	15	00
Denaturation	94 ºC	00	30
Annealing	58 ºC	00	30
Extension	72 ºC	01	00
Final Extension	72 ºC	05	00
Cycles	30		

4.3 Results

4.3.1 Programmed Cell Death (PCD) assays by flow cytometry

Flow cytometry was used to determine the contribution of PCD to the cytotoxic effects of live, heat-killed, BCM of (ESBL-ve *E. coli* as control and ESBL-ve *E. coli* bacterial strains) and the CM of *Acanthamoeba* on TERT-NHUC cell line. Data from the flow cytometry images (Figure 4.1, 4.4, 4.7, and 4.10), were extrapolated into graphs for analysis.

4.3.1.1 Programmed Cell Death assays on TRET-NHUC line exposed to live bacteria

This study analysed the effects of visible clinical strains of *E. coli* (ESBL +ve, and ESBL–ve as a control) on urothelial cell line apoptosis and necrosis assays. As displayed in Figure 4.1, unviable cells were determined to be in their early apoptotic, late apoptotic and necrotic stages through differential staining. Early apoptotic cells were positive for Annexin-FITC and negative for Zombie NIR. By comparison, while late apoptotic cells were positive for both Annexin-FITC and Zombie NIR, necrotic cells were positive for Zombie NIR alone. Viable cells were negative for both Annexin-FITC and Zombie NIR. As displayed in Figures 4.2 and 4.3, urothelial cells employed both apoptotic and necrotic pathways for cell death over 24h due to cytotoxicity that was induced by live bacteria (10 or 100 bacteria/cell). The most significant flow cytometric analysis of live bacteria (Figures 4.2 and 4.3) revealed that all the bacterial strains induced a higher cytotoxic effect on urothelial cells at an infectious dose of 10 bacteria/cell than at an infectious dose of 100 bacteria/cell. In addition, the OXA-48 and TEM strains were more toxic to the urothelial cells than the other strains for both infectious doses (10 and 100 bacteria/cell). As illustrated by differential staining, cell populations that included early apoptotic cells (Figures 4.2 and 4.3) included all studied strains and revealed that low rates of toxicity were less than 0.5%. Cells in the late apoptotic stages (Figures 4.2 and 4.3) were

significantly highest with the TEM strain with ten bacteria/cell (1.2%), followed by the AmpC and OXA-48 strains with ten bacteria/cell (1.05%). Cells in the late apoptotic stages were shown with the OXA-48 strain with 100 bacteria/cell (1.05%), followed by the TEM and AmpC strains with 100/bacteria cell (0.4%). Moreover, in infectious doses of 10 bacteria/cell, the OXA-48 strain induced the significantly highest percentage of necrosis (6.5%), the AmpC strain induced the second highest rate of necrosis (5.7%) and the TEM strain induced the lowest percentage of necrosis (5.35%). By comparison, infectious doses of 100 bacteria/cell, the OXA-48 strain induced highest percentage of necrosis (5.7%) significantly, the TEM strain induced the second significantly the highest percentage of necrosis (3.5%) and the AmpC strain induced the lowest (2.75%).

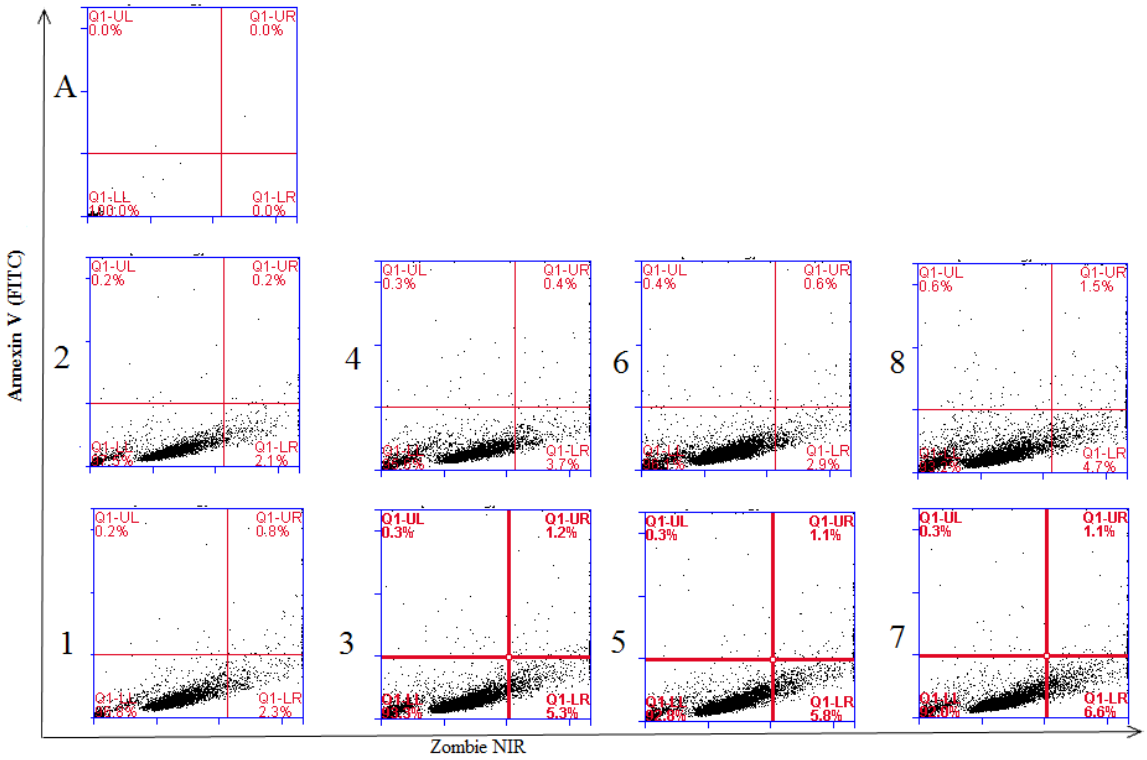

Figure 4.1: Cytotoxic effects of visible strains of *E. coli* (ESBL+ve and ESBL–ve).

This is an image showing viable, early apoptotic, late apoptotic and necrotic cells. Apoptosis and necrosis were induced on TERT-NHUC line by live bacteria over 24h of incubation. In each panel, the lower left quadrant shows viable cells (LL - negative for both Zombie NIR and Annexin V), the lower right quadrant shows necrosis (LR - Zombie NIR positive cells). The upper right quadrant shows late apoptosis (UR - positive for both Zombie NIR and Annexin V), and the upper left quadrant shows early apoptosis (UL - Zombie NIR positive cells). Images were obtained from FlowJo (v3.05470) and are representative of results obtained from at least three independent experiments.

A – Cells without live bacteria (control), 1 to 8 – cells with live bacteria. 1- *E. coli-ve* (10 bacteria/cell). 2- *E. coli-ve* (100 bacteria/cell). 3- TEM (10 bacteria/cell). 4- TEM (100 bacteria/cell). 5- AmpC (10 bacteria/cell). 6- AmpC (100 bacteria/cell). 7- OXA-48 (10 bacteria/cell). 8- OXA-48 (100 bacteria/cell).

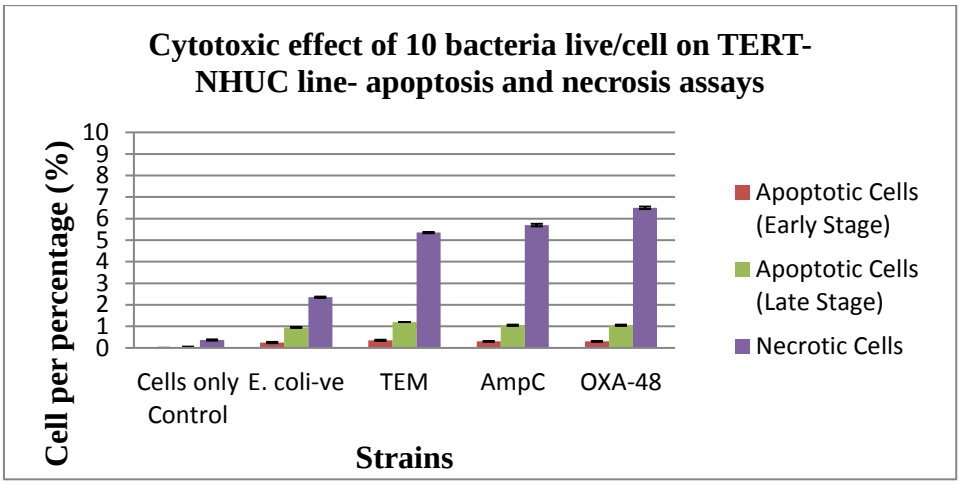

Figure 4.2: Cytotoxic effects of 10 bacteria live/cell on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic and necrotic cells were extrapolated from flow cytometry images. The percentages of the viable cell are not shown in this figure. Results are obtained from at least three independent experiments that were performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

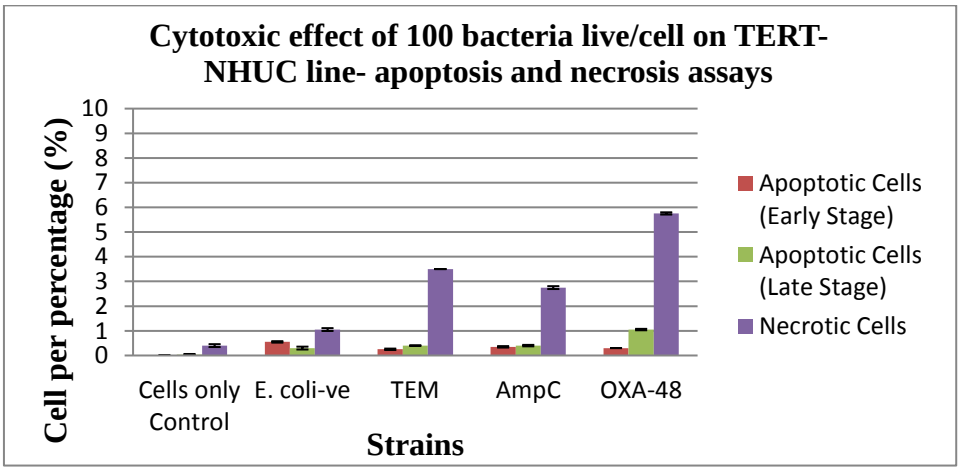

Figure 4.3: Cytotoxic effects of 100 bacteria live/cell on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic and necrotic cells were extrapolated from flow cytometry images. The percentages of the viable cells are not shown in this figure. Results are obtained from at least three independent experiments performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.1.2 Programmed Cell Death (PCD) assays for TRET-NHUC line exposed heat-killed bacteria

This study analysed the effects of Heat-killed strains of *E. coli* (ESBL +ve and ESBL–ve) on urothelial cell line apoptosis and necrosis assays. As displayed in Figure 4.4, unviable cells were determined to be in their early apoptotic, late apoptotic and necrotic stages through differential staining. Early apoptotic cells were positive for Annexin-FITC and negative for Zombie NIR. By comparison, while late apoptotic cells were positive for both Annexin-FITC and Zombie NIR, necrotic cells were positive for Zombie NIR alone. Viable cells were negative for both Annexin-FITC and Zombie NIR. As displayed in Figures 4.5 and 4.6, urothelial cells employed both apoptotic and necrotic pathways for cell death over 24h due to cytotoxicity that was induced by heat-killed bacteria (10 or 100 bacteria/cell). The most significant flow cytometric analysis of heat-killed bacteria (Figures 4.5 and 4.6) revealed that all the bacterial strains induced a higher cytotoxic effect on urothelial cells at an infectious dose of 100 bacteria/cell than at an infectious dose of 10 bacteria/cell. In addition, OXA-48 and TEM strains were more toxic to the urothelial cells than the other strains for both infectious doses (10 or 100 bacteria/cell). All strains displayed significant ($P<0.05$ using t-test, one-tailed distribution) than the cytotoxic effect of the ESBL-ve strain on the cells. As illustrated by differential staining, cell populations that included early apoptotic cells (Figures 4.5 and 4.6) revealed that low rates of toxicity were less than 0.55%, except AmpC strain was 0.7% with infectious dose 10 bacteria/cell. Cells in the late apoptotic stages (Figures 4.5 and 4.6) were significantly highest with OXA-48 strain using 10 bacteria/cell (0.95%). AmpC strain was significantly lower than the ESBL-ve with 10 bacteria/cell (0.55%).

OXA-48 strain had significantly late apoptotic effects when using 100 bacteria/cell (2.05%), and AmpC strains (1.2%) respectively then TEM strain with 100/bacteria cell (0.4%). Moreover, in infectious doses of 10 bacteria/cell, TEM strain induced highest percentage of

necrosis (3.95%) significantly, the OXA-48 strain induced the second significantly the highest percentage of necrosis (3.5%). By comparison, in infectious doses of 100 bacteria/cell, the AmpC strain induced the highest percentage of necrosis (5.7%) significantly, the OXA-48 strain induced the second significantly the highest percentage of necrosis (5.5%).

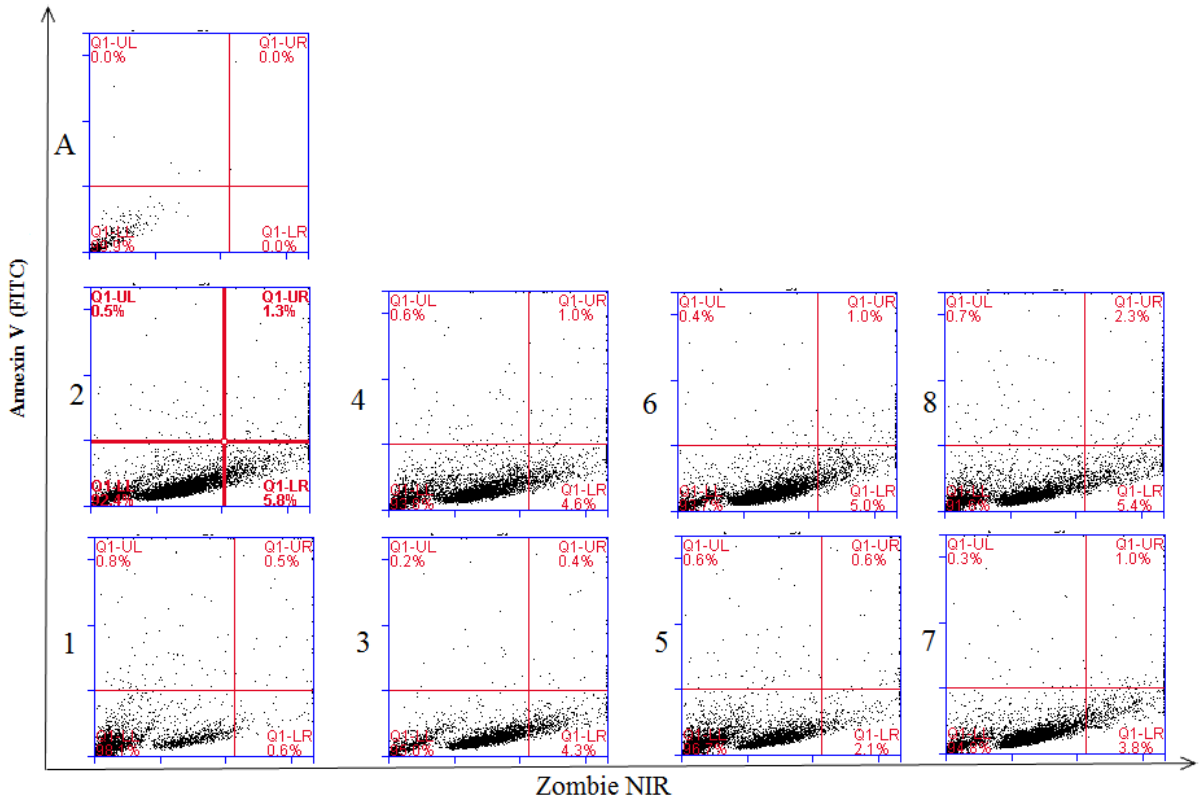

Figure 4.4: Cytotoxic effects of heat-killed bacteria strains of *E. coli* (ESBL+ve and ESBL–ve).

This is an image showing viable, early apoptotic, late apoptotic and necrotic cells. Apoptosis and necrosis were induced on TERT-NHUC line by heat-killed bacteria over 24h of incubation. In each panel, the lower left quadrant shows viable cells (LL - negative for both Zombie NIR and Annexin V), the lower right quadrant shows necrosis (LR - Zombie NIR positive cells). The upper right quadrant shows late apoptosis (UR - positive for both Zombie NIR and Annexin V), and the upper left quadrant shows early apoptosis (UL - Zombie NIR positive cells). Images were obtained from FlowJo (v3.05470) and are representative of results obtained from at least three independent experiments.

A – Cells without live bacteria (control), 1 to 8 – cells with heat killed bacteria. 1- *E. coli-ve* (10 bacteria/cell). 2- *E. coli-ve* (100 bacteria/cell). 3- TEM (10 bacteria/cell). 4- TEM (100 bacteria/cell). 5- AmpC (10 bacteria/cell). 6- AmpC (100 bacteria/cell). 7- OXA-48 (10 bacteria/cell). 8- OXA-48 (100 bacteria/cell).

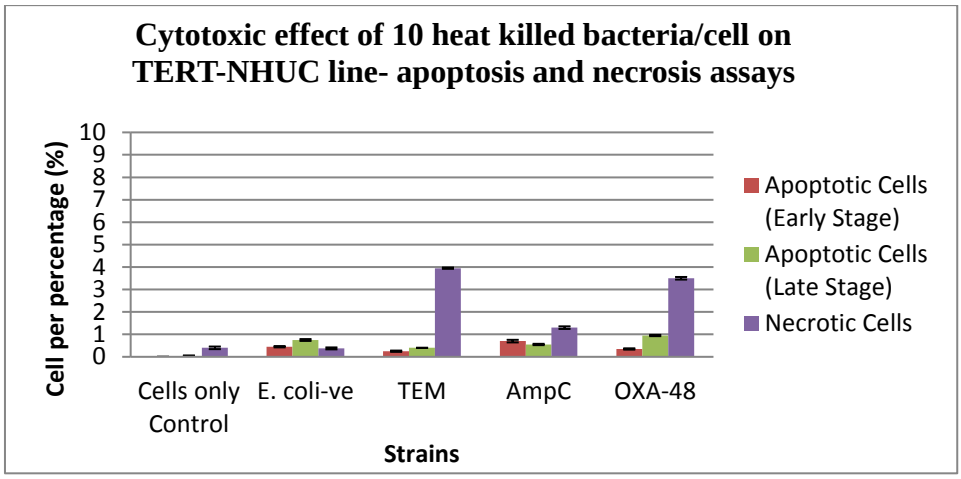

Figure 4.5: Cytotoxic effects of 10 heat-killed bacteria/cell on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic and necrotic cells were extrapolated from flow cytometry images. The percentages of the viable cells are not shown in this figure. Results are obtained from at least three independent experiments that were performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

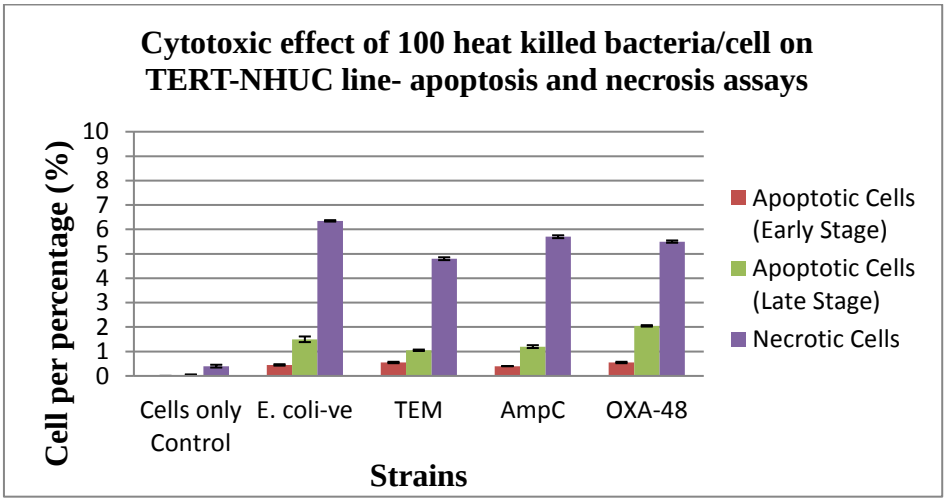

Figure 4.6: Cytotoxic effects of 100 heat-killed bacteria/cell on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic and necrotic cells were extrapolated from flow cytometry images. The percentages of the viable cells are not shown in this figure. Results are obtained from at least three independent experiments performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.1.3 Programmed Cell Death (PCD) assays for the CMB on TRET-NHUC line

Bacterial Conditioned Media (BCM) were prepared to investigate the potential roles of enzymes, toxins and proteases produced by *E. coli* (ESBL +ve and ESBL–ve) in apoptosis and necrosis assays. Unviable cells were determined to be in early apoptotic, late apoptotic or necrotic stages through differential staining, as shown in Figure 4.7. Early apoptotic cells were positive for Annexin-FITC and negative for Zombie NIR, late apoptotic cells were positive for both Annexin-FITC and Zombie NIR, and necrotic cells were only positive for Zombie NIR. Moreover, viable cells were found to be negative for both Annexin-FITC and Zombie NIR. As depicted in Figures 4.8 and 4.9, urothelial cells followed both apoptotic and necrotic pathways to cell death due to the cytotoxicity induced by BCM (at doses of 10 or 100 bacteria/cell) over a 24h incubation period. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test. The most special from a flow cytometric analysis of BCM (Figures 4.8 and 4.9) revealed that at infectious doses of 10 bacteria/cell, all bacterial strains induced a less cytotoxic effect on urothelial cells than they did at infectious doses of 100 bacteria/cell. In addition, OXA-48 strains induced more cytotoxicity on urothelial cells than do the other strains and the control (ESBL-ve) at both infectious doses. Following infection of cells with all the strains, differential staining showed that less than 0.45% of the cells were in the early stage of apoptosis (Figures 4.8 and 4.9). Contrastingly, toxicity was highest with the OXA-48 strain at both infection doses of 10 and 100 bacteria/cell (0.55% and 0.65%, respectively). The highest proportions of cells in late apoptotic stages with infection doses of 10 bacteria/cell were significantly found in the AmpC strain (0.95%) and TEM strains (0.75% for both). Furthermore, the most cells in upper late apoptotic stages at the infection dose of 100 bacteria/cell were significantly found in the OXA-48 strains (1.7%, respectively) and the AmpC strain (0.9%). At the infection dose of 10 bacteria/cell, AmpC strains induced the highest percentage of necrosis significantly (4.5%), followed by the TEM strain (4.1%). At the infection dose of 100

bacteria/cell, the ESBL –ve (6.13%) were significantly highest percentages of necrosis than the other ESBL+ve strains, as shown in Figures 4.8 and 4.9.

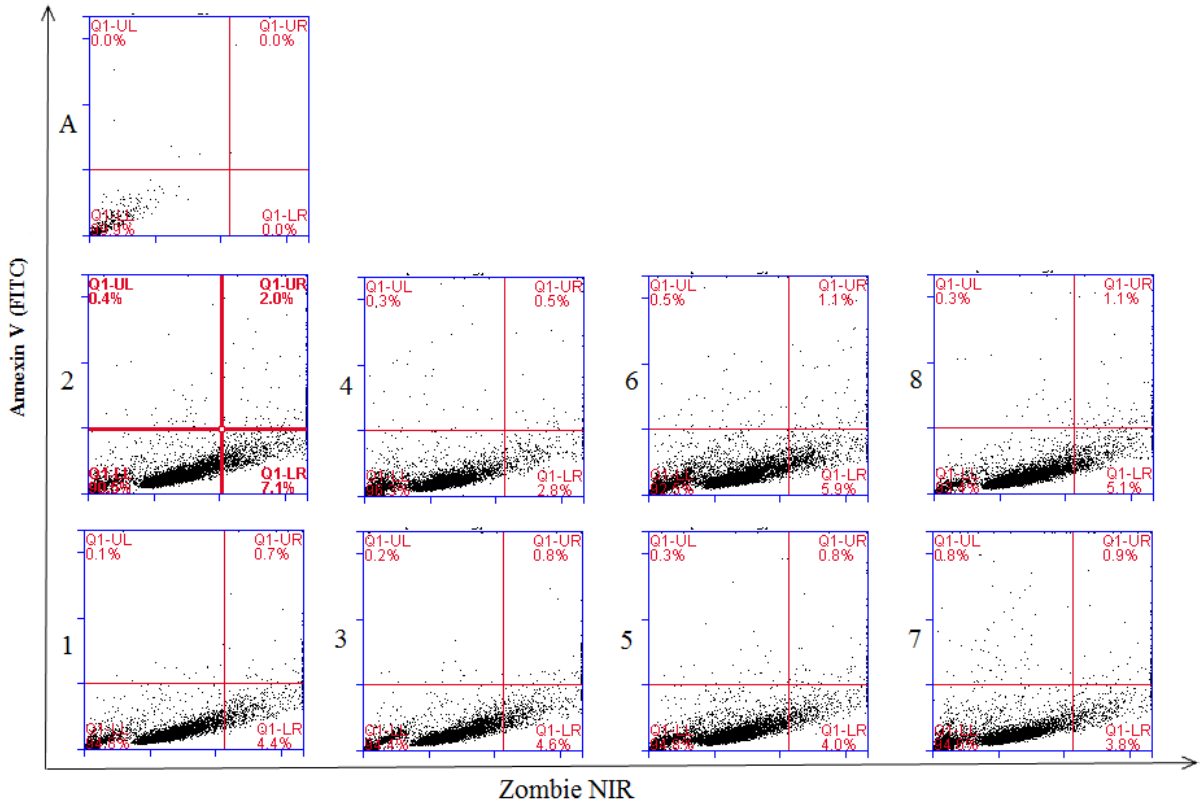

Figure 4.7: Cytotoxic effects of BCM of *E. coli* (ESBL+ve and ESBL–ve).

This is an image showing viable, early apoptotic, late apoptotic and necrotic cells. Apoptosis and necrosis were induced on TERT-NHUC line by BCM over 24h of incubation. In each panel, the lower left quadrant shows viable cells (LL - negative for both Zombie NIR and Annexin V), the lower right quadrant shows necrosis (LR - Zombie NIR positive cells). The upper right quadrant shows late apoptosis (UR - positive for both Zombie NIR and Annexin V), and the upper left quadrant shows early apoptosis (UL - Zombie NIR positive cells). Images were obtained from FlowJo (v3.05470) and are representative of results obtained from at least three independent experiments.

A – Cells without live bacteria (control), 1 to 8 – cells with BCM. 1- *E. coli-ve* (10 bacteria/cell). 2- *E. coli-ve* (100 bacteria/cell). 3- TEM (10 bacteria/cell). 4- TEM (100 bacteria/cell). 5- AmpC (10 bacteria/cell). 6- AmpC (100 bacteria/cell). 7- OXA-48 (10 bacteria/cell). 8- OXA-48 (100 bacteria/cell).

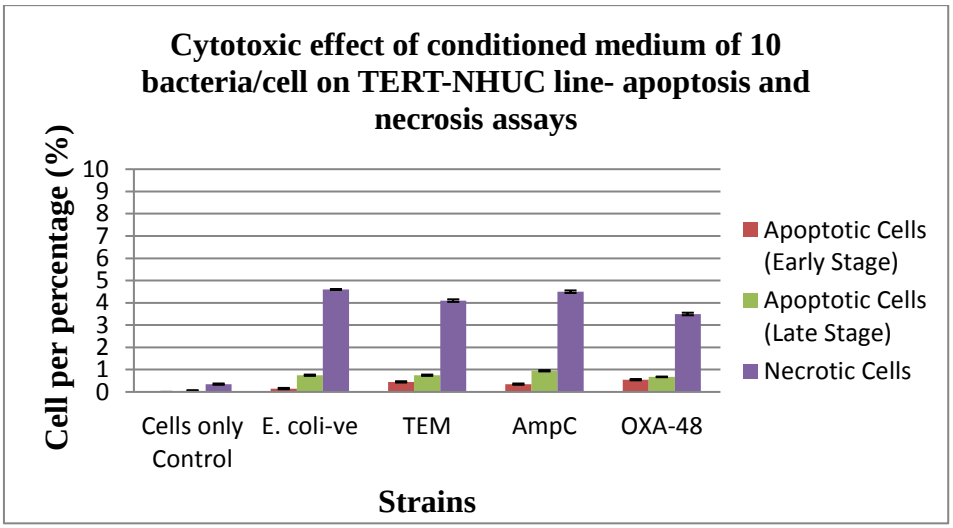

Figure 4.8: Cytotoxic effects of BCM of 10 bacteria/cell on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic, and necrotic cells were extrapolated from flow cytometry images. The percentages of viable cells are not shown in this figure. Results are obtained from at least three independent experiments that were performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

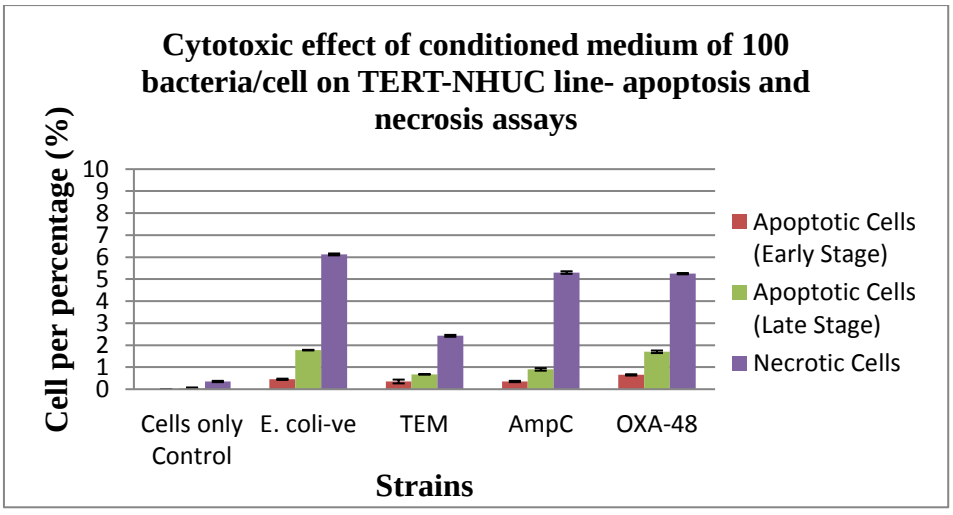

Figure 4.9: Cytotoxic effects of BCM of 100 bacteria/cell on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic, and necrotic cells were extrapolated from flow cytometry images. The percentages of viable cells are not shown in this figure. Results are obtained from at least three independent experiments that were performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.1.4 Programmed Cell Death (PCD) assays of ACM on TRET-NHUC line

ACM was prepared to investigate whether the enzymes, toxins and proteases produced by *Acanthamoeba* have roles in apoptosis. Unviable cells were determined to be in early apoptotic, late apoptotic or necrotic stages through differential staining, as shown in Figure 4.10. Early apoptotic cells were positive for Annexin-FITC and negative for Zombie NIR, late apoptotic cells were positive for both Annexin-FITC and Zombie NIR, and necrotic cells were positive only for Zombie NIR. Moreover, viable cells were negative for both Annexin-FITC and Zombie NIR. As displayed in Figures 4.11 and 4.12, urothelial cells followed both apoptotic and necrotic pathways of cell death due to the cytotoxicity induced by ACM (10µl and 100µl volumes) over a 24h period. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test. The most interesting finding reveals that ACM in volumes of 100µl can induce higher cytotoxicity on urothelial cells than can ACM volumes of 10µl. As shown in the differential staining results in Figures 4.11 and 4.12, none of the cells was in the early apoptotic stage. However, more cells in late apoptotic stages were significantly higher found in ACM (100µl; 0.4%) than in ACM (10µl; 0.15%). Moreover, ACM 10µl volumes revealed a significantly lower percentage of necrosis (0.067%) than did doses at 100µl (0.65%), as shown in Figures 4.11 and 4.12.

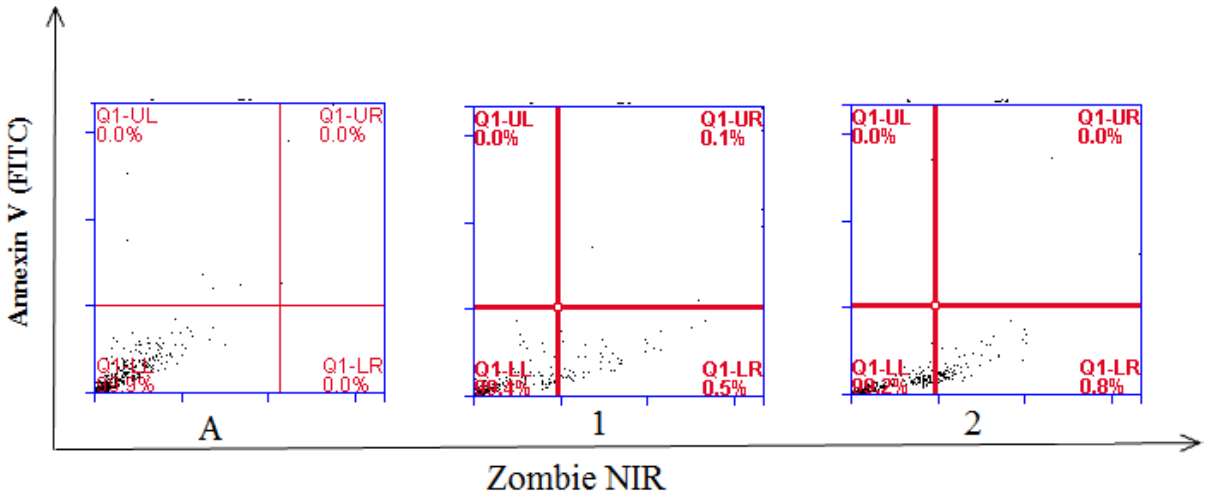

Figure 4.10: Cytotoxic effects of ACM.

This is an image showing viable, early apoptotic, late apoptotic and necrotic cells. Apoptosis and necrosis were induced on TERT-NHUC line by ACM over 24h of incubation. In each panel, the lower left quadrant shows viable cells (LL - negative for both Zombie NIR and Annexin V), the lower right quadrant shows necrosis (LR - Zombie NIR positive cells). The upper right quadrant shows late apoptosis (UR - positive for both Zombie NIR and Annexin V), and the upper left quadrant shows early apoptosis (UL - Zombie NIR positive cells). Images were obtained from FlowJo (v3.05470) and are representative of results obtained from at least three independent experiments.

A – Cells without ACM (control), 1- ACM (10μl). 2- ACM (100μl).

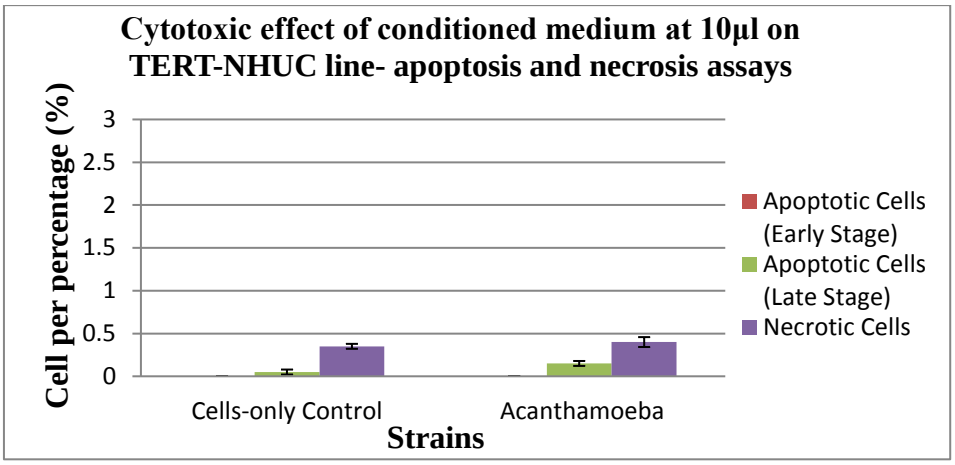

Figure 4.11: Cytotoxic effects of ACM at 10µl on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic, and necrotic cells were extrapolated from flow cytometry images. The percentages of viable cells are not shown in this figure. Results are obtained from at least three independent experiments that were performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

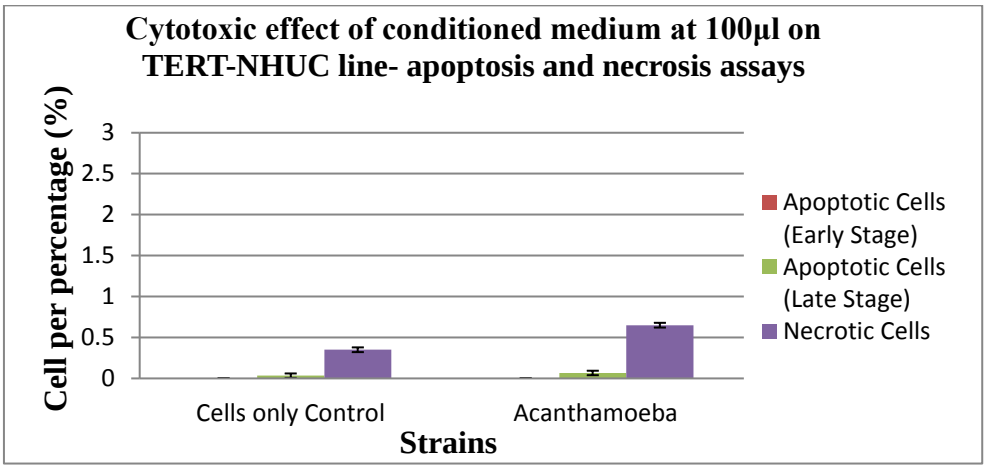

Figure 4.12: Cytotoxic effects of ACM at 100µl on TERT-NHUC line.

The percentages of early apoptotic, late apoptotic, and necrotic cells were extrapolated from flow cytometry images. The percentages of viable cells are not shown in this figure. Results are obtained from at least three independent experiments that were performed in duplicate. Error bars represent the standard error. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.2 LDH assays

In order to further differentiate cytotoxicity induced by uropathogenic bacteria (live, heat-killed bacteria, BCM, and ACM) LDH assays were performed to determine the percentage of cytotoxicity produced on TERT-NHUC line.

4.3.2.1 Cytotoxic effects of live bacteria on TRET-NHUC line

Cytotoxicity assays of live bacteria were performed to check the pathogenicity of ESBL +ve strains and using ESBL-ve as a control on TRET-NHUC line. In these assays, visible bacteria were used, and results were compared with the ESBL –ve strain. A low and higher number of bacteria (10 or 100 bacteria/cell) were used to determine whether this will have any impact on bacterial cytotoxicity after 24h incubation.

In general, unexpectedly, increased number of bacteria showed decreased cytotoxic effect on the cell line. For example, the cytotoxicity of 10 live bacteria/cell after 24h were (TEM was 13.56%, AmpC was 12.93%, and OXA-48 was 15.52%), however, the cytotoxicity of 100 live bacteria/cell after 24h were (TEM was 7.09%, AmpC was 6.08%, and OXA-48 was 7.84%). These values are significantly higher than the cytotoxic effect of the live ESBL-ve (control) isolate on the cells ($P<0.05$ using a paired t-test, one-tailed distribution). All ESBL+ve bacteria showed a significant effect on cell line compared with the ESBL–ve (Figure 4.13).

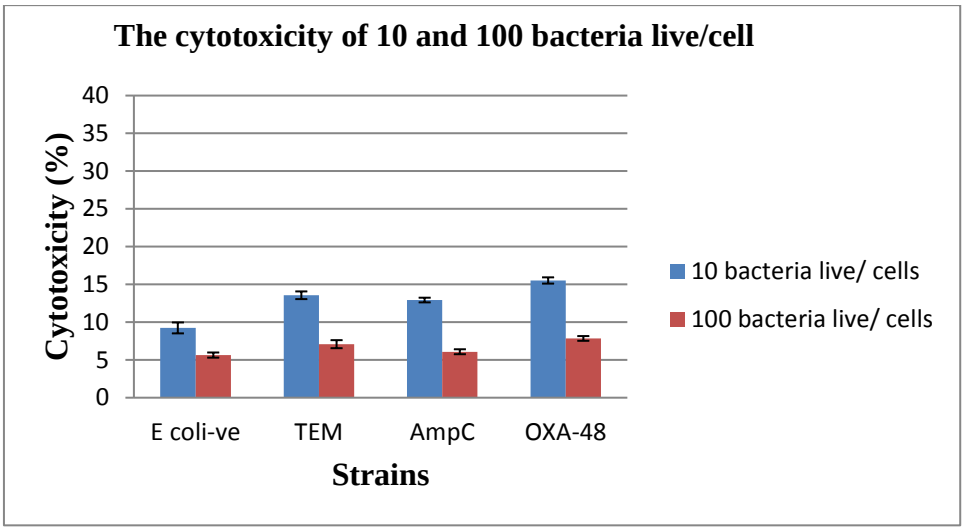

Figure 4.13 Cytotoxic effects of live bacteria on TRET-NHUC line at 10, 100 bacteria/cell after 24h. These results are the mean of three independent experiments carried out in duplicate. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.2.2 Cytotoxic effects of heat-killed bacteria on TRET-NHUC line

To investigate whether the factors were causing cytotoxicity to have protein nature, cytotoxicity assays were performed using heat-killed bacteria of ESBL +ve strains on TRET-NHUC and using ESBL-ve *E. coli* as a control. In these assays, heat-killed bacteria were used, and results were compared with the ESBL –ve strain. A low and higher number of bacteria (10 or 100 bacteria/cell) were used to determine whether this will have any impact on bacterial cytotoxicity after 24h incubation.

The results showed that the increased number of bacteria showed an increased cytotoxic effect on the cell line. For more details, the cytotoxicity of 10 heat-killed bacteria/cell after 24h were significantly higher with ESBL+ve (TEM 5.77%, AmpC 5.92%, and OXA-48 5.33%) than the cytotoxic effect of the ESBL-ve strain (3.16%) on the cells. In addition, the cytotoxicity of 100 live bacteria/cell after 24h were significantly higher with ESBL+ve (TEM 13.08%, AmpC

11.15%, and OXA-48 16.07%) than the cytotoxic effect of the ESBL –ve strain (9.41%). These values are significantly higher than the cytotoxic effect of the heat-killed ESBL-ve (control) isolate on the cells ($P<0.05$ using a paired t-test, one-tailed distribution). All ESBL+ve bacteria showed a significant effect on cell line compared with the ESBL–ve (Figure 4.14).

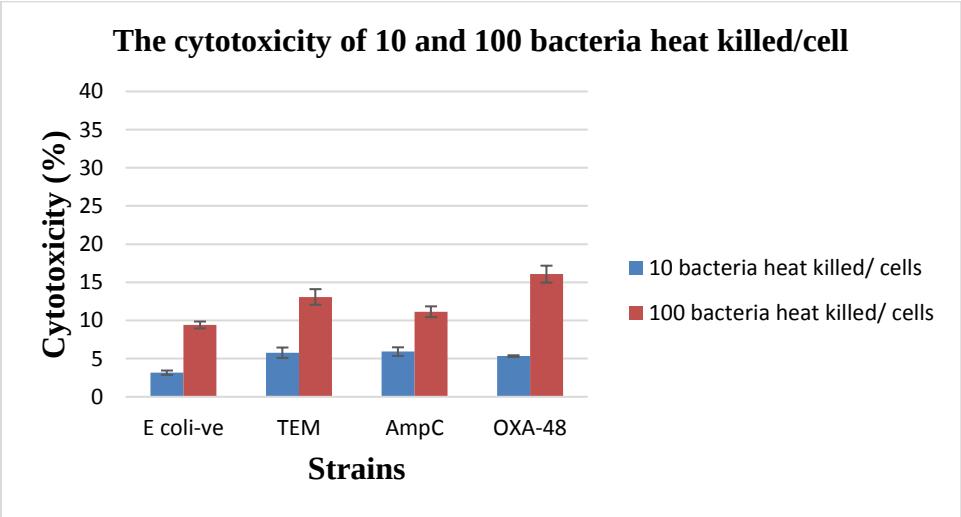

Figure 4.14 Cytotoxic effects of heat-killed bacteria on TRET-NHUC line at 10, 100 bacteria/cell doses after 24h. These results are the mean of three independent experiments carried out in duplicate. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.2.3 Cytotoxic effects of the BCM on the TRET-NHUC line

Cytotoxicity assays of bacterial conditioned media were performed to confirm the pathogenicity of the effects of ESBL +ve bacteria on the TRET-NHUC line compared with the control ESBL –ve *E. coli.* BCM, as prepared in section 4.2.2.2, was used to determine whether the bacteria contents of the conditioned media notably, proteins have any impact on cell line cytotoxicity after 24h incubation.

The results showed that increasing the number of bacteria/cell produced a greater cytotoxic effect on the cell line. As seen in Figure 4.15, in doses of 10 bacteria/cell, the

cytotoxicity of TEM was 4.71%, in AmpC was 23.44%, in OXA-48 was 7.67%, and in ESBL−ve was 3.47%. However, the cytotoxicity induced by doses of 100 bacteria/cell after 24h of TEM was 17.73%, in AmpC was 32.20%, in OXA-48 was 10.2%, and in ESBL −ve was 9.22%. This was noted through a calculated $p < 0.05$ value for BCM using a paired t-test with a one-tailed distribution. All ESBL +ve bacteria indicated a significant effect on the cell line compared with the ESBL –ve bacteria (see Figure 4.15).

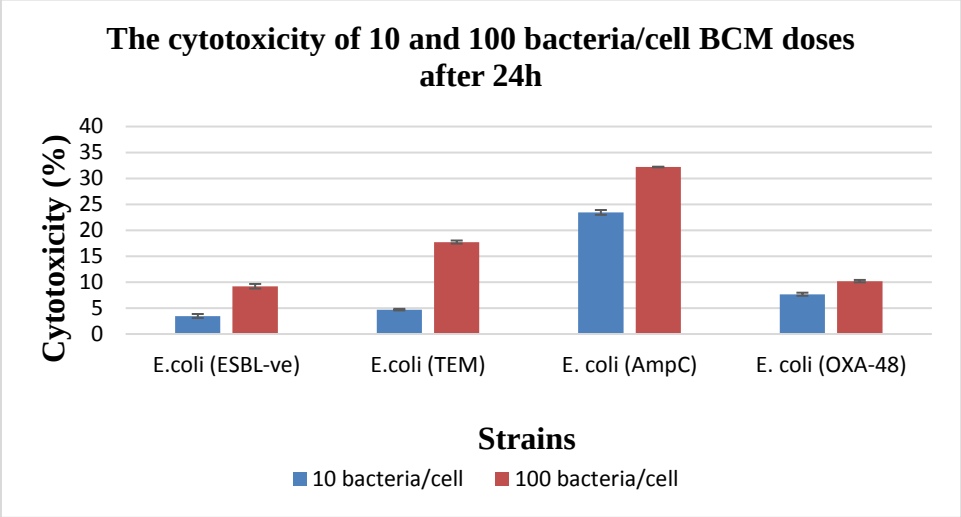

Figure 4.15 Cytotoxic effects of the BCM on the TRET-NHUC line at 10 and 100 bacteria/cell doses after 24h. These results are the mean of three independent experiments carried out in duplicate. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.2.4 Cytotoxic effects of ACM on the TRET-NHUC line

Cytotoxicity assays were performed to assess the pathogenicity of conditioned ACM (T4) on the TRET-NHUC line. ACM, as prepared in section 4.2.3.1, was used (10µl and 100µl volumes) to determine whether the ACM would have an impact on *Acanthamoeba* cytotoxicity after a 24h incubation.

In general, unexpectedly, volumes of 100µl revealed a more significant cytotoxic effect on the cell line than did volumes of 10µl. After 24h, the cytotoxicity of ACM at volumes of 100µl was found to be 5.77%; however, the cytotoxicity of ACM at volumes of 100µl was observed to be 2.44%. This was noted as $p < 0.05$; the value was calculated through a paired t-test with a one-tailed distribution (Figure 4.16).

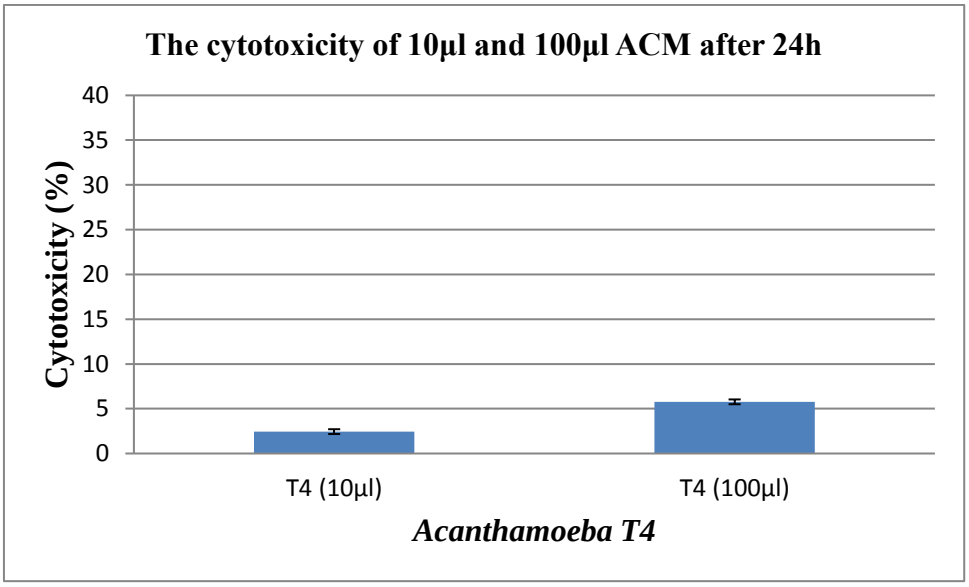

Figure 4.16 Cytotoxic effects of the ACM on the TRET-NHUC line at 10µl and 100µl after 24h. These results are the mean of three independent experiments carried out in duplicate. P values of < 0.05 were accepted to be significantly different based on the paired one-tailed t-test.

4.3.3 Polymerase Chain Reaction (PCR)

Results of this study showed dissimilarity in the virulence factors borne by the ESBL strains under study (Table 4.3). The ESBL strains were investigated for *E. coli* virulence factors (VF), using an updated version (personal correspondence from Prof. James Johnson, 2013) of Johnson and Stell's PCR assays (Johnson and Stell, 2000). Five sets of primers (hlyD, CNF-1, pic, sat, and kbsII (Kii)) were used to study the virulence factors amplified from the genomic DNA of the strains under study (ESBL-ve and ESBL +ve (TEM, AmpC, and OXA-48). All the

strains under study are not seen to bear on the hlyD, CNF-1, and pic genes. The PCR products from the kbsII (Kii) Primer to ESBL –ve *E. coli*, TEM, AmpC, and OXA-48 were observed, and corresponded to the expected results, with approximately 570 bp were successfully amplified, as seen in (Figure 4.17). The most important in these results that TEM and AmpC strains were observed to have Sat gene with corresponding to the expected result, with approximately 937 bp were successfully amplified, and this gene was absent from the other strains under study.

Table 4.3: Summary of virulence factors amplified from the genomic DNA of the strains under study (ESBL-ve and ESBL+ve (TEM, AmpC, and OXA-48).

HlyA is lysing erythrocytes allowing the producing strain to obtain iron for growth and survival in the absence of siderophores. The CNF1 is generating multinucleated cells that develop into necrotic lesions. Sat is a toxin interferes with the cytoskeleton of epithelial cells and acts as a serine protease. Pic plays a role in lysing mucin as part of diarrheal disease and mediates serum resistance. The essential functions of Kbsll are to facilitate the invasion of tissues and evasion of the host immune system.

	HlyD	CNF-1	Sat	pic	Kbsll
ESBL-ve	-	-	-	-	+
TEM	-	-	+	-	+
AmpC	-	-	+	-	+
OXA-48	-	-	-	-	+

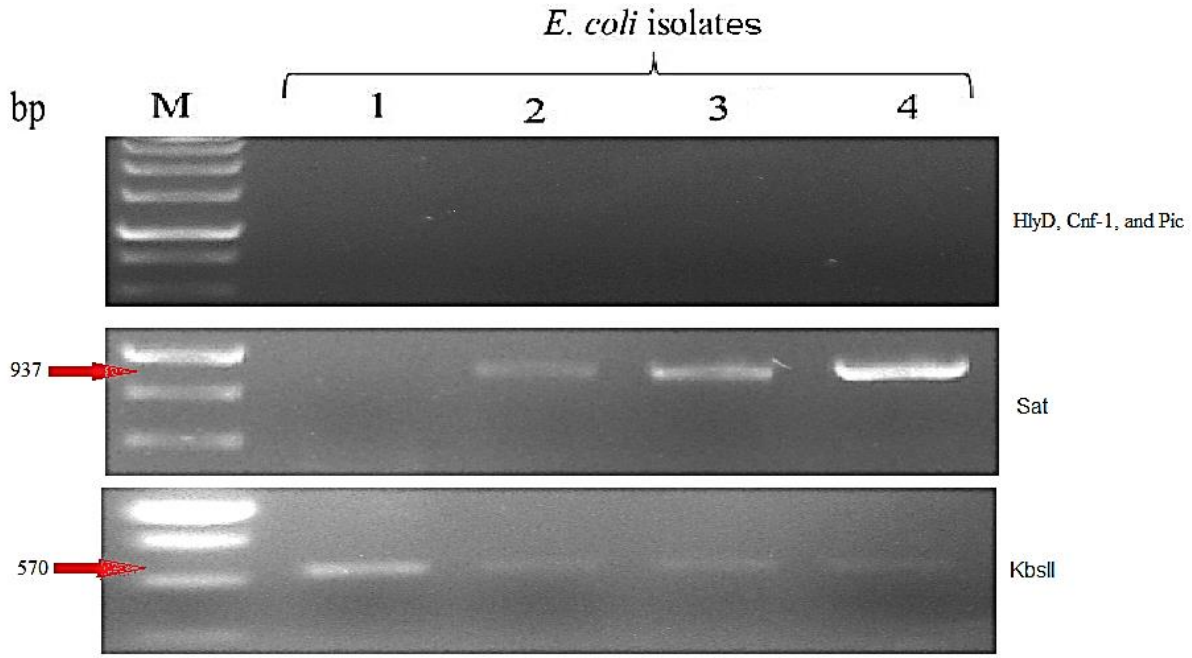

Figure 4.17: PCR products amplified using hlyD, CNF-1, pic, sat, and kbsII (Kii) primers. Bands were observed in Sat and KbsII lanes with corresponded near to the expected (Sat (937bp) and KbsII (570bp). M: Marker. Lanes 1-*E. coli* (ESBL –ve), 2-TEM, 3-AmpC and 4-OXA-48.

4.4 Discussion

This study set out with the aim of exploring the cytotoxicity among uropathogens bacteria and *Acanthamoeba* onto TERT-NHUC cell line. Flow cytometry assays and cytotoxicity assays by (LDH) were used to determine the contribution of PCD and the cytotoxic effects of live and heat-killed of (ESBL positive *E. coli* bacterial strains and ESBL-ve *E. coli* as a control) and the CM of bacteria and *Acanthamoeba* on TERT-NHUC cell line by using two different doses (10 and 100 bacteria or amoeba/cell).

There are two forms of cell death in infections: apoptosis and necrosis. Cell death forms are involved in infection as an immune response to clear the infection area by macrophages (Serra *et al.*, 2015). Moreover, it is well known that microorganisms have a role in cell death. However, each organism has different virulence factors, including contact-dependent factors like pili and flagella, also has contact-independent factors such as α-toxin and CNF-1 (Worgall *et al.*, 2002; Haslinger-Loffler *et al.*, 2005; Khan, 2009).

Bacterial pathogens can also induce apoptosis and in some cases host cell necrosis (Kim *et al.*, 2008). Flow cytometry assays conducted in the present study revealed evidence of TERT-NHUC apoptosis and necrosis induced by live, heat killed, and conditioned medium of all bacteria under investigation and *Acanthamoeba* (Figure 4.1 to 4.12).

A high percentage of necrosis was seen with the low number of live bacteria. All live bacterial strains induced a higher rate of late apoptosis and necrosis on urothelial cells with 10 bacteria/cell than at an infectious dose of 100 bacteria/cell. Moreover, data from LDH assays Figure 4.13 were in line with flow cytometry results in Figure 4.2 and 4.3. For live bacteria, the OXA-48 and TEM strains were significantly more apoptotic and necrotic with the urothelial cells compared with the control with both infectious doses (10 and 100 bacteria/cell). In addition, with infectious doses of 10 bacteria/cell, OXA-48 strain induced the highest percentage of late

apoptosis and necrosis (7.55%) compared with the control (0.40%) and the other ESBL+ve strains (8% and 6.75% for TEM and AmpC respectively). On the other hand, in infectious doses of 100 bacteria/cell, OXA-48 strain induced significantly the highest percentage of late apoptosis and necrosis (6.75%), the TEM strain induced the second highest percentage of late apoptosis and necrosis (3.9%) compared with the control (0.43%). Finally, cytotoxicity assays with LDH revealed an increased number of bacteria and a decreased cytotoxic effect on the cell line. For example, the cytotoxicity of 10 live bacteria/cell was significantly the highest after 24h with OXA-48 strain (15.52%) compared with the control (9.24 %) and other ESBL+ve strains (13.56% and 12.93% for TEM and AmpC respectively). Also, the cytotoxicity of 100 live bacteria/cell was also the highest with OXA-48 strain (7.84%) than the control, (Figure 4.13). Previous studies (mostly uropathogenic *E. coli*) have documented the interaction of bacteria with NHUC. Thus, contributes to the pathogenesis of urinary tract infection (Bower *et al.*, 2005; Wiles *et al.*, 2008; Bang *et al.*, 2014). Bacterial pathogens, therefore, induce apoptosis and in some cases host cell necrosis (Kouokam *et al.*, 2006; Wiles *et al.*, 2008; Garcia *et al.*, 2013; Smith *et al.*, 2015).

The most significant findings from the flow cytometric analysis of heat-killed bacteria (as seen in Figures 4.5 and 4.6) revealed that at infectious doses of 10 bacteria/cell, all bacterial strains induced less death in urothelial cells than did infectious doses of 100 bacteria/cell. Moreover, data from LDH assays Figure 4.14 were in line with flow cytometry results in Figure 4.5 and 4.6. For heat-killed bacteria, OXA-48 strain was significantly more apoptotic and necrotic on the urothelial cells compared with other ESBL+ve strains and the control with both infectious doses (10 and 100 bacteria/cell). Furthermore, the most cells of late apoptotic and necrosis stages at infectious doses of 10 bacteria/cell were significantly found with OXA-48 strain (4.9%) and the TEM strain (4.30%). On the other hand, doses of 100 bacteria/cell resulted that most cells of late apoptotic and necrosis stages with OXA-48 strain (7.55%) and AmpC strain (6.9%). Cytotoxicity assays by LDH showed that the higher dose of bacteria-induced an

increased cytotoxic effect on the cell line. In particular, the cytotoxicity induced by doses of 10 heat-killed bacteria/cell was significantly highest with ESBL+ve (AmpC (5.92%), TEM (5.77%) and OXA-48 (5.33%)) compared with the control. Also, the highest toxicity scores resulting from doses of 100 bacteria/cell were significantly found with ESBL+ve (OXA-48 (16.07%), TEM (13.08%) and AmpC (11.15%)) compared with the control. Many studies have documented the effect of heat-killed bacteria. Heat-killed bacteria can trigger substantial molecular responses and cause genomic instability (Koturbash *et al.*, 2009). The interaction of the body bacteria with NHUC contributes to the pathogenesis of urinary tract infection (Wiles *et al.*, 2008; Bang *et al.*, 2014). Heat-killed bacteria showed immune-modulating effects that pro-inflammatory cytokine production in the cells and affected intestinal metabolism (Sugahara *et al.*, 2017).

The most special from flow cytometric analysis of the BCM (Figure 4.8 and 4.9) confirms that at infectious doses of 10 bacteria/cell, all bacterial strains induce a less cytotoxic effect on urothelial cells than do infectious doses of 100 bacteria/cell. Moreover, data from LDH assays Figure 4.15 were in line with flow cytometry results in Figure 4.8 and 4.9. BCM at infectious doses of 10 bacteria/cell, AmpC strain (5.45%) was more apoptotic and necrotic on the urothelial cells compared with the ESBL-ve control (5.35%). However, ESBL-ve (control) (7.91%) was more apoptotic and necrotic with the urothelial cells compared with the ESBL+ve (OXA-48 (6.95%) and AmpC (6.20%). Finally, the results of cytotoxicity assays by LDH showed that the higher dose of bacteria-induced an increased cytotoxic effect on the cell line. The cytotoxicity of the 10 and 100 bacteria/cell doses were significantly higher with AmpC strain (23.44% and 32.20%, respectively) compared with the control ESBL-ve (3.47% and 9.22%, respectively) and other ESBL+ve strains. These observations support the concept that toxins, enzymes and proteases produced by pathogens may have a significant role in inducing cytotoxic effects on cells (McIlwain *et al.*, 2013). Moreover, *E. coli* K1 conditioned medium showed anti-amoebic effects (Iqbal *et al.*, 2014). It is evident from these results that

uropathogens bacteria have exhibited severe cytotoxic effects on NHUC. The results showed that the toxic effect of the conditioned medium of all the strains used on TRET-NHUC line was higher with more number of bacteria compared with the low number of bacteria after 24h.

The higher toxicity rate observed in the OXA-48 strain can also be attributed to horizontal gene transfer which always occurs between *E. coli* strains (Juhas, 2015). This is because OXA-48 may have acquired virulence genes which enhance its toxicity (van Duin and Doi, 2017). As was the case in this study, the OXA 48 strain was seen to bear plasmid-borne CTX-M and TEM genes, that code for β-lactamases, both of which are known to play roles in the virulence of OXA 48 (Cuzon *et al.*, 2011).

Overall, the results from the current study suggest that uropathogens bacteria have the ability to induce significant cytotoxicity during UTIs. Necrotic responses of human urothelial cells to uropathogens bacteria were dependent on BCM, as they were higher than live bacteria and heat-killed bacteria. Also, the other most interesting finding is that the infectious dose of 10 bacteria/cell for live bacteria has a higher cytotoxic effect than an infectious dose of 100 bacteria/cell. However, with an infectious dose of 10 bacteria/cell for BCM and heat-killed bacteria, all the bacterial strains induced a less cytotoxic effect on urothelial cells than at an infectious dose of 100 bacteria/cell.

Findings from this experiment have revealed that ACM volumes of 100µl induce greater cell death on urothelial cells than ACM volumes of 10µl. Moreover, data from LDH assays Figure 4.16 were in line with flow cytometry results Figure 4.11 and 4.12. As shown by the differential staining results in Figures 4.11 and 4.12, more cells in the late apoptotic and necrosis stages were found following ACM volumes of 100µl (1.05%) than following ACM volumes of 10µl (0.22%). Additionally, the results of the cytotoxicity assays by LDH showed that ACM volumes of 100µl induced a more significant cytotoxic effect on the cell line than did ACM

volumes of 10µl. The exact compilation of ACM is unknown, but in a recent study, Iqbal et al., (2014) have indicated the presence of active components in the range of 5 to 10kDa. Different studies have proved the ability of *Acanthamoeba* trophozoites and their lysate to cause cell death (Zheng *et al.*, 2004; Chusattayanond *et al.*, 2010). Some types of *Acanthamoeba* trophozoites produce Ecto-ATPases, which hydrolyse the extracellular adenosine triphosphate (ATP) to adenosine diphosphate (ADP). ADP will be released as a result of *Acanthamoeba* binding with host cells causing caspase-3 activation and then apoptosis (Khan, 2009). *Acanthamoeba* can release a diversity of extracellular factors like proteases into their environment, either as a protective mechanism or as part of their normal physiological process. As a result, these released factors can potentially have a significant effect on other microorganisms living in the same environment. In these finding, we found that *Acanthamoeba* by ACM 100µl can induce cell death on urothelial cells higher than ACM 10µl. This finding supports the previous study, which links extracellular *Acanthamoeba* proteases to the degradation of the extracellular matrix (Sissons *et al.*, 2006; Retana-Moreira *et al.*, 2015).

In conclusion, Monolayer cultures were used in the investigations of the present study. Conversely, the human urothelium is made of 3 layers, and the primary urothelial mediated response to infections and toxicity is the superficial cell exfoliation and regeneration of the urothelium (Nagamatsu *et al.*, 2015). Thus, it is hard to inference these *in vitro* results to the urinary tract. For further studies must be taken these variables into account. Nevertheless, the results from this chapter should provide the basis for further investigations, especially *in vivo*, to clarify the toxicity of uropathogens bacteria on human urothelial cells.

In the present study, we compared several virulence genes which belong to (the toxins separated and capsule) between four clinical strains of *E. coli* (ESBL +ve and ESBL–ve as a control). Results obtained from this study showed a variation in the virulence factors borne by the ESBL strains under study (Table 4.1). The ESBL strains were investigated for *E. coli* VFs by

using five sets of primers (hlyD, CNF-1, pic, sat, and kbsII (Kii)) (Johnson and Stell, 2000; Rodriguez-Siek *et al.*, 2005). PCR amplification was carried out on the chromosomal DNA of all *E. coli* strains under investigation.

Pathogenic *E. coli* strains, cause different infections, including UTIs. One of these reasons to help these strains to initiate diseases it is their virulence factors that may play a role in infection by enabling the bacterial to attach, invade the host and disseminate. *E. coli* VFs include adhesion molecules and toxins (Mora-Rillo, Fernández-Romero, Navarro-San Francisco, *et al.*, 2015). Once attachment to host cells have been started, *E. coli* secrete toxins that work to damage host cells and destroy host tissues; completing the second stage of infection. The most important in these results, AmpC and TEM strains were seen to have *Sat* gene and this gene absence from the other strains under study. Secreted autotransporter toxin (Sat) is dominant Extra-intestinal pathogenic *E. coli* (ExPEC) toxin, which had been identified in 39%-94% of investigated strains (Pitout *et al.*, 2005; Johnson *et al.*, 2008; Mahjoub-Messai *et al.*, 2011). This toxin interferes with the cytoskeleton of epithelial cells and acts as a serine protease (Guyer *et al.*, 2000; Dobrindt *et al.*, 2002). Furthermore, the PCR products from the *kbsII* (Kii) gene were seen to bear in all the strains under investigation (Figure 4.17). The primary function of *E. coli* capsules, are to facilitate the invasion of tissues and evasion of the host immune system (Ananias and Yano, 2008). The majority of ExPEC strains are capsulated, supporting the idea of capsules as an essential virulence factor (Whitfield and Roberts, 1999; Ovchinnikova *et al.*, 2016). By using the PCR method in the present study, it was demonstrated that AmpC and OXA-48 strains are highly toxic more virulent and resistant than the other strains under study. From this outcome, we can not generalise these findings to all strains as only three strains were used in this project. In future, we may have different results using enough strains for statistical analysis.

The next chapter explores the antimicrobial efficacy of CPC; that is primarily used to treat oral bacterial infections. Many studies have investigated the use of CPC in oral care but not UTIs.

CHAPTER FIVE 5

5. The effects of cetylpyridinium chloride (CPC) on the uropathogenic bacteria, *Acanthamoeba*, and the urothelial cell line

5.1 Introduction

Multidrug-resistant strains possessing Extended-Spectrum β-Lactamases (ESBLs) have become an increasing problem all over the world. ESBLs are specific enzymes that can break down many types of antibiotics, which leads to their inactivation. ESBLs cause global health problems since infections caused by enzyme-producing organisms are associated with high morbidity and mortality rates and the financial burden for treatment can be costly. β- lactamases are hydrolytic enzymes which cleave the β- lactam ring conferring bacterial resistance to β-lactam antibiotics, like penicillins and cephalosporins (Dhillon and Clark, 2012). IBCs formed inside bladder urothelial cells are associated with recurrent UTIs (Robino *et al.*, 2013; Glover *et al.*, 2014; Scott *et al.*, 2015). These IBCs allow the bacteria to avoid the host immune response (Anderson *et al.*, 2004) also IBCs offer them resistance to antibiotics (Hannan *et al.*, 2012). It is well known that major antibiotic resistance is exhibited by bacteria found within intracellular bacterial communities (Stewart and Costerton, 2001).

In 2009, a study concluded that *Acanthamoeba* was found in 26% of urine samples under investigation (Santos *et al.*, 2009). *Acanthamoeba* is a protozoan which feed on bacteria and can act as a trojan horse for them. Another previous study showed that the pathogenic *E. coli* K1 interact with *Acanthamoeba* and has the ability to invade and remain viable inside *Acanthamoeba*. In addition, invasive *E. coli* K1 is able to enter human macrophages, survive and replicate intracellularly (Alsam *et al.*, 2006). The presence of *Acanthamoeba* in certain patients may be helping pathogenic bacteria in urine, protecting them against antimicrobial drugs, disinfectants, and host immunity. Inside *Acanthamoeba*, the pathogenic multi-drug resistant bacteria survive and replicate (Cardas *et al.*, 2012). It was discovered that the pathogenic bacteria become more virulent and more resistant to antibiotics if they infect an amoeba and then emerge from it (Moon *et al.*, 2011). Re-recurrence of UTIs is very common, with 27% of patients suffering another infection within six months and 44% of patients experiencing another infection

within one year. Between one-third and one-half of the recurrent infections are caused by the same strain that initiated the disease. Thus, the recurrent infections will help the bacteria to become more resistant to antimicrobials (Hilbert, 2013).

The development of increased resistance against available antibiotics is a major global concern and is a significant threat to the successful treatment of infectious diseases. Therefore, looking for new or additional methods and compounds for treatment is urgent.

Quaternary ammonium compounds (QACs) are cationic surfactants used as ingredients in a variety of household and industrial products. Many types of QACs have a significant use as antibacterial, antifungal and antiviral abilities, and they are an increasingly promising area of research (Jiao *et al.*, 2017). Cetylpyridinium Chloride (CPC) belongs to the group of QACs. Most studies researched the effect of CPC only on oral care (Hwang *et al.*, 2013) and poultry processing (Arritt *et al.*, 2002; Beers *et al.*, 2006). Considering its useful antimicrobial activity and its cost-effectiveness, CPC can be investigated for its possible use in treating UTIs. By using CPC for treatment of both ESBL+ve strains and *Acanthamoeba* may help many patients suffering from prolonged and recurrent of UTIs.

This chapter has been divided into three parts. The first part deals with the antimicrobial effects of CPC on bacteria. Secondly, cytotoxicity assays were performed to explore the cytotoxic effects of CPC on *Acanthamoeba* and TERT-NHUC line. Then the last part deals with antimicrobial intracellular amoebic and urothelial cell survival assays.

5.2 Materials and methods

All the chemicals for this study were bought from Sigma Laboratories and Thermofisher unless otherwise stated.

5.2.1 Cell line and cell culture

Normal Human Urothelial Cell Line (TRET-NHUC) was grown and cultured as mentioned in section (3.2.3).

5.2.2 Bacterial culture

All strains were cultured onto CLED plates and incubated at 37^{o}C overnight. A few colonies were inoculated in LB broth, and the following day, ODs were measured by using spectrophotometer as explained in chapter 2 (2.2.1).

5.2.3 *Acanthamoeba* culture

Acanthamoeba T4 strain was grown and cultured as described in chapter 3 (3.2.2).

5.2.4 Artificial Urine Medium (AUM)

AUM was prepared based on the analysis of human urine constituents (Brooks and Keevil, 1997) as mentioned in chapter 3 (3.2.4).

5.2.5 CPC as an antimicrobial agent

A stock solution (1mg/ml) of CPC (Sigma C0732) was made in distilled water and stored at RT no longer than one month. Different concentrations (1.5, 3, 5, and 10μg/ml) of CPC were prepared from the stock solution for use in the experiments.

5.2.5.1 CPC as an antibacterial agent

To determine the effects of CPC on bacteria (*E. coli* (ESBL –ve), TEM, AmpC, and OXA-48) CPC was prepared (Zarei *et al.*, 2013). Firstly, a stock solution (1mg/ml) was made in distilled water and stored at RT. Four different concentrations (1.5, 3, 5, and 10 μg/ml) of CPC were prepared from the stock solution for use in the experiments. Two different infectious doses of bacterial strains (~1×10^6 CFU/ml and ~1×10^7 CFU/ml) were used in this investigation. Bacterial cultures were grown in AUM with different concentrations of CPC in a 24-well plate. The plates were incubated at 37°C for 24h, diluted and cultured onto CLED agar plates and the colonies were counted after 18h of incubation at 37°C.

5.2.5.2 CPC as antiamoebic agent

To determine the cytotoxic effect of CPC on *Acanthamoeba* (T4) CPC was prepared as above. *Acanthamoeba* trophozoites from confluent flasks (~ 95%) were detached by placing the flasks on ice for 20min. The separated *Acanthamoeba* in PYG were seeded (~ 5x 10^6 cells per ml) in a 24-well plate and incubated at 30°C overnight. Next day, each well was washed once with PBS and fresh AUM was added (control) or AUM with CPC (1.5, 3, 5, and 10μg/ml) and the plates were incubated for 24h at 30°C in an incubator. After incubation, amoebae viability was determined by adding 0.1% trypan blue and the number of live (non-stained) and dead (stained) amoebae were counted by using a haemocytometer.

5.2.6 Effects of CPC on intracellular bacteria

5.2.6.1 Effects of CPC on intracellular bacteria inside *Acanthamoeba*

Intracellular survival assays of uropathogens bacteria inside *Acanthamoeba* were performed as described in section (3.2.5.3). However, after killing the extracellular bacteria, each well was washed with PBS, and fresh AUM (control) or medium with CPC (1.5, 3, 5, and 10µg/ml) were added, and the plates were incubated for 24h at 30°C. After incubation, each well was washed once with PBS and the *Acanthamoeba* were lysed using cell lytic (Sigma C3228-500ml) (150µl). Bacteria were quantified onto CLED agar plates (Alsam *et al.*, 2006).

5.2.6.2 Effects of CPC on intracellular bacteria inside urothelial cell line

Intracellular survival assays of uropathogenic bacteria in TERT-NHUC line were performed as described in section (3.2.5.3). However, after killing the extracellular bacteria, each well was washed with PBS, and fresh KGM-2 (control) or medium with CPC (1.5, 3, 5, and 10µg/ml) were added, and the plates were incubated for 24h at 37°C in humidified 95% air and 5% CO_2. After incubation, each well was washed once with PBS and the cells were lysed using cell lytic (150µl). Bacteria were quantified onto CLED agar plates (Alsam *et al.*, 2006).

5.2.7 Cytotoxicity assays by LDH

To determine the cytotoxic effects of CPC on TERT-NHUC line CPC was used. Medium was discarded from T25 flasks with confluent TERT-NHUC line, and the cells were washed with 2ml PBS. Accutase (0.5ml) was added, and the flasks were incubated at 37°C for 5min to detach the cells after which the detached cells were resuspended in KGM-2 and centrifuged 1000 x g for 5min. The supernatants were discarded, and the cells were resuspended in fresh KGM-2 medium and passaged into 24-well plates and incubated at 37°C in humidified 95% air and 5% CO_2

incubator. Next day, each well was washed once with PBS and supplemented with fresh KGM-2 (control) and KGM-2 with CPC (1.5, 3, 5, and10μg/ml). The plates were incubated for 24h at 37°C in humidified 95% air and 5% CO_2 incubator. After the incubation period, LDH assays were performed as described in chapter 4 (4.2.5).

5.2.8 Software and statistics

Microsoft Office Excel programme (v15.30) was used for general data analysis to make graphs and to calculate the means, standard deviation and standard error. To find the significant differences between bacterial strains, the one-tailed t-test was used to calculate P-value.

5.3 Results

5.3.1 CPC as an antimicrobial agent

5.3.1.1 CPC as an antibacterial agent

Antibacterial assays were performed to determine the antimicrobial effects of CPC on bacterial uropathogens. Four different concentrations of CPC (1.5, 3, 5, and 10μg /ml) were tested for their antimicrobial efficacy over a period of 24h. A paired one-tailed t-test was used to analyse the relationship between the control (bacterial growth in AUM only) and uropathogenic bacteria under study at two different infectious doses (~1×10^6 CFU/ml and ~1×10^7 CFU/ml) of bacteria.

Firstly, from the figures below (Figure 5.1 to Figure 5.4) we can see that CPC has displayed potent antimicrobial effects at sufficient concentrations. Secondly, CPC antimicrobial activity exhibited positive correlation with the number of bacteria, a decrease in bacterial growth with an increase in concentration. At a concentration of 1.5μg/ml, CPC has significantly ($P<0.05$) reduced bacterial growth to less than 50% whereas an 80% decrease ($P<0.05$) was seen at a concentration of 3μg/ml. In addition, there was a meagre percentage of bacterial growth (0.00041%) at a concentration of 5μg/ml CPC. At 10μg/ml, CPC has wholly killed all uropathogenic bacteria (Figure 5.1 to 5.4). As seen in Figure 5.1 in the presence of 1.5μg/ml of CPC for an infecting dose of 10 bacteria /cell, the ESBL+ve strains of *E. coli* (TEM (36.59% survival), AmpC (38.89% survival), and OXA-48 (54.70% survival)) have a higher percentage of survival compared with their ESBL negative counterparts (34.91% survival). Furthermore, the presence of 1.5μg/ml of CPC for an infecting dose of 100 bacteria/cell with ESBL+ve strains (TEM (33.57% survival), AmpC (33.66% survival), and OXA-48 (49.25% survival)) have a higher percentage of survival compared with their ESBL negative counterparts (22.91%). However, the effect of 1.5μg/ml of CPC on an infecting dose of 100 bacteria/cell was less than its effect on an infection dose of 10 bacteria/cell. As evident in Figure 5.2, the CPC has fully

inhibited bacterial growth at both infectious doses at a concentration of 3µg/ml, in all cases except that of OXA-48 at an infecting dose of 100 bacteria/cell (when 17.21% of bacteria survived). Moreover, at a concentration of 5µg/ml, CPC has fully inhibited bacterial growth at both infectious doses except for OXA-48 at infecting dose of 100 bacteria/cell where survival was (0.00041%) as seen in Figure 5.3. These findings reveal that 10µg/ml of CPC fully inhibited OXA-48 (Figure 5.4).

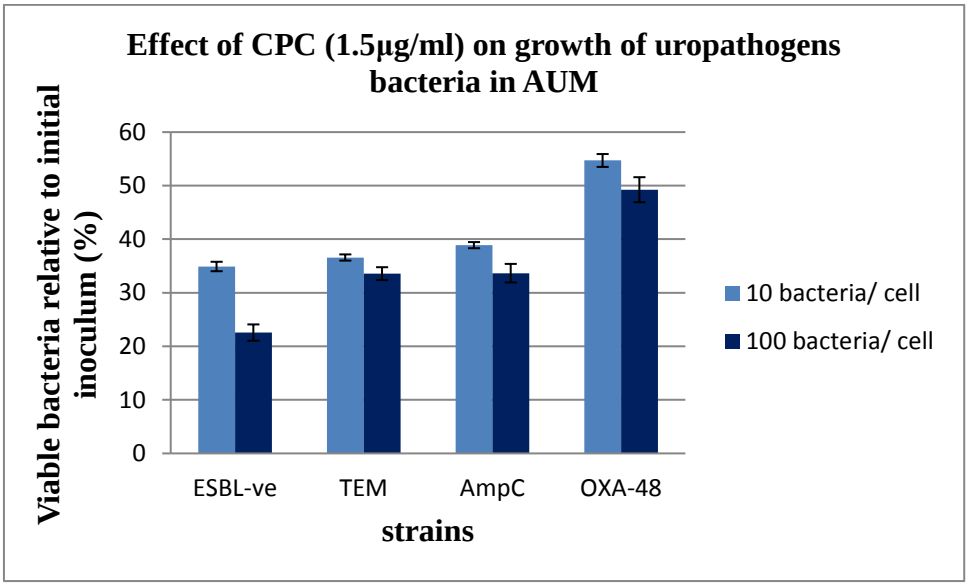

Figure 5.1 Effect of CPC (1.5µg/ml) on the growth of uropathogenic bacteria with two different infecting doses of bacterial strains (~1×10^6 CFU/ml (10 bacteria/cell) and ~1×10^7 CFU/ml (100 bacteria/cell)) in AUM. Viable bacteria were counted after 24h of incubation at 37°C. The percentage growth is calculated by comparing growth in AUM with CPC and AUM without CPC (control). Results are the mean of three independent experiments carried out in duplicate. Error bars symbolise standard error. Results are significant at $P<0.05$ (paired one-tailed t-test).

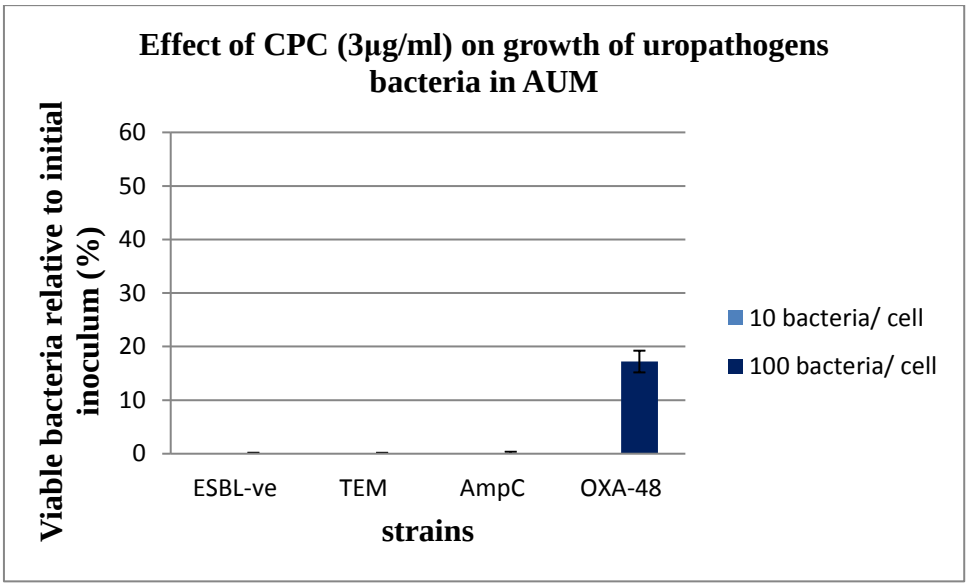

Figure 5.2 Effect of CPC (3µg/ml) on the growth of uropathogenic bacteria with two different infecting doses of bacterial strains (~1×10^6 CFU/ml (10 bacteria/cell) and ~1×10^7 CFU/ml (100 bacteria/cell)) in AUM. Viable bacteria were counted after 24h of incubation at 37°C. The percentage growth is calculated by comparing growth in AUM with CPC and AUM without CPC (control). Results are the mean of three independent experiments carried out in duplicate. Error bars symbolise standard error. Results are significant at P<0.05 (paired one-tailed t-test).

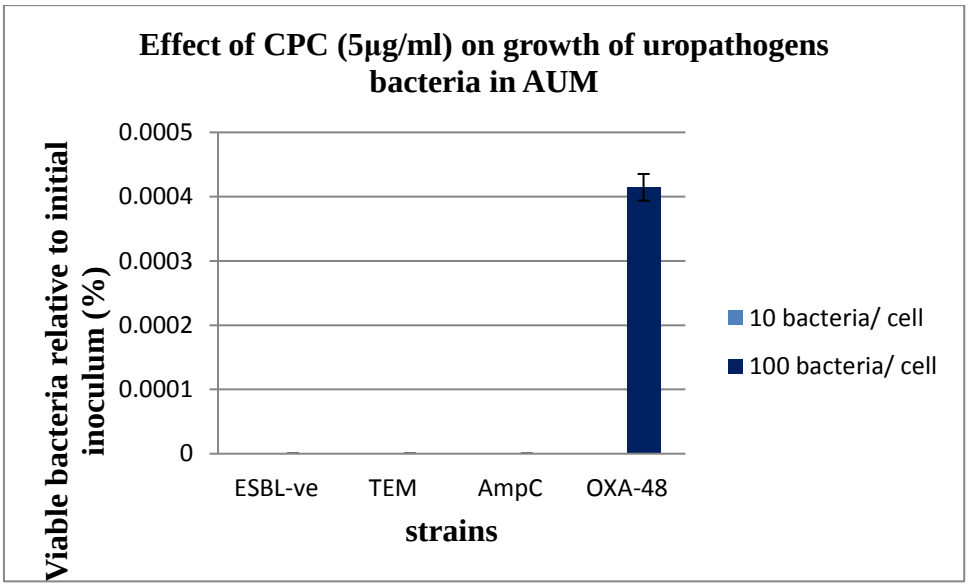

Figure 5.3 Effect of CPC (5µg/ml) on the growth of uropathogenic bacteria with two different infecting doses of bacterial strains (~1×10^6 CFU/ml (10 bacteria/cell) and ~1×10^7 CFU/ml (100 bacteria/cell)) in AUM. Viable bacteria were counted after 24h of incubation at 37°C. The percentage growth is calculated by comparing growth in AUM with CPC and AUM without CPC (control). Results are the mean of three independent experiments carried out in duplicate. Error bars symbolise standard error. Results are significant at $P<0.05$ (paired one-tailed t-test).

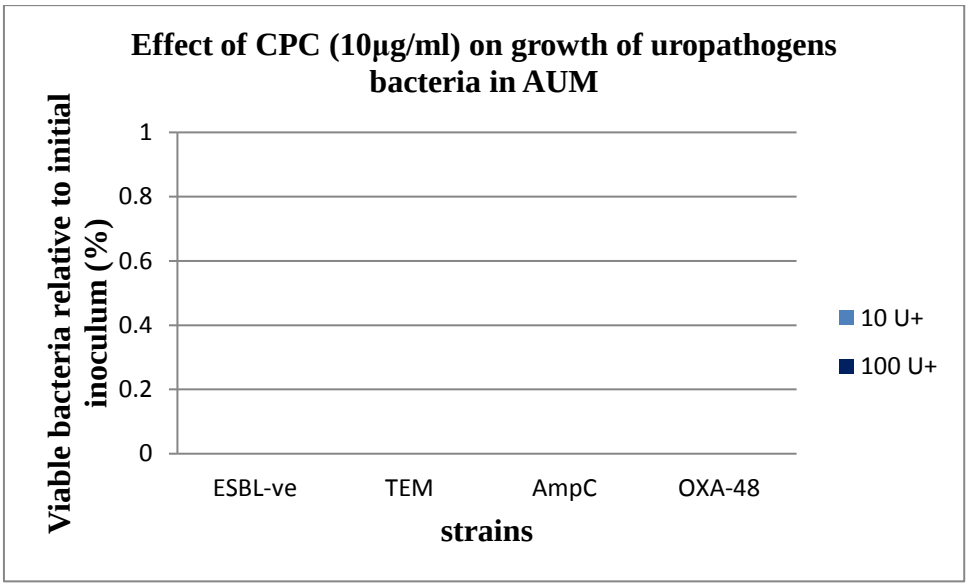

Figure 5.4 Effect of CPC (10µg/ml) on the growth of uropathogenic bacteria with two different infecting doses of bacterial strains (~1×10^6 CFU/ml (10 bacteria/cell) and ~1×10^7 CFU/ml (100 bacteria/cell)) in AUM. Viable bacteria were counted after 24h of incubation at 37°C. The percentage growth is calculated by comparing growth in AUM with CPC and AUM without CPC (control). Results are the mean of three independent experiments carried out in duplicate. Error bars symbolise standard error.

5.3.1.2 CPC as antiamoebic agent

Trypan blue viability assays were performed to determine the activity effect of CPC on *Acanthamoeba* (T4) over 24h. The cytotoxicity was measured based on differential staining; dead amoebae take up the trypan blue stain, and the live amoebae remain (non-stained).

As shown in Figure 5.5, CPC at the concentrations tested has failed to induce sufficient levels of cytotoxicity in amoebae. However, CPC antiamoebic activity increased with an increase in concentration. The percentage of cytotoxicity is low for all tested concentrations of CPC 1.5, 3, 5, and 10µg/ml (0.35%, 1.07%, 1.63%, and 6.65% respectively) as seen in Figure 5.5. It should also be noted that higher concentrations of CPC (>10µg/ml) caused the amoebae to detach from the surface and turn to cysts.

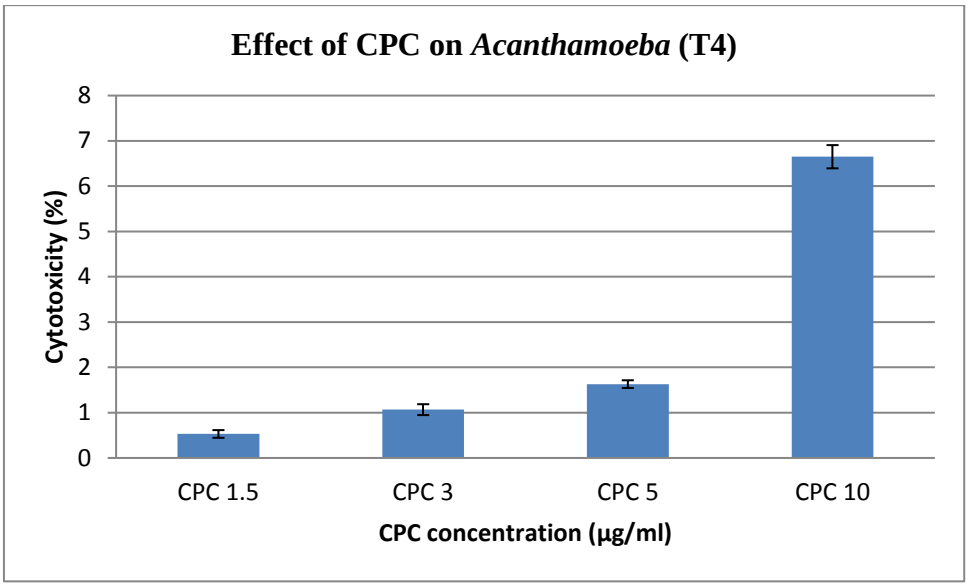

Figure 5.5 The cytotoxicity effect of CPC on *Acanthamoeba* (T4).

Amoebae were incubated with CPC for 24h and cytotoxicity was assayed using trypan blue. Percentage cytotoxicity relative to control cells is shown. CPC 1.5 - 1.5µg/ml; CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at P<0.05 (paired one-tailed t-test).

5.3.2 Effects of CPC on intracellular bacteria

5.3.2.1 Effects of CPC on intracellular bacteria in *Acanthamoeba*

Intra-amoebic survival assays were performed to determine the ability of CPC to kill intracellular bacteria. The experiment was conducted on the OXA-48 strain, because this was the only strain found to survive and multiply inside the *Acanthamoeba*. *Acanthamoeba* T4 invaded with bacteria were incubated at 30°C for 24h. The bacteria surviving within *Acanthamoeba* (T4) were calculated as follows: number of viable bacteria recovered (CFU/ml) / number of bacteria in initial inoculum x 100 (Alsam *et al.*, 2006; Yousuf *et al.*, 2013). As seen in Figure 5.6 and 5.7, four different concentrations of CPC were tested using two different infecting doses of OXA-48 in *Acanthamoeba* T4. A paired one-tailed t-test was used to analyse the relationship between the

control (OXA-48 strain survival without CPC) and OXA-48 strain (different infecting dose of bacteria) with different concentrations of CPC (1.5, 3, 5, and 10µg /ml)).

These results showed that the increased concentrations of CPC correlated with a decreased level of survival of the bacteria. As evident in Figure 5.6, CPC has displayed potent antimicrobial effects at low concentrations. At infecting doses of 10 bacteria/amoeba, a concentration of 1.5µg/ml, CPC significantly ($P<0.05$) reduced bacterial growth to 0.075%, of the control, and at a concentration of 3µg/ml to 0.048% of the control. Very low levels of survival were found at a CPC concentration of 5µg/ml (0.026%) with no surviving bacteria at a concentration of 10µg/ml. Additionally, the concentrations of 1.5µg/ml, 3µg/ml, 5µg/ml, and 10µg/ml of CPC gave very low levels of survival when the infecting inoculums was 100 bacteria per amoeba (0.076%, 0.046%, 0.029%, and 0.001% respectively) (Figure 5.7).

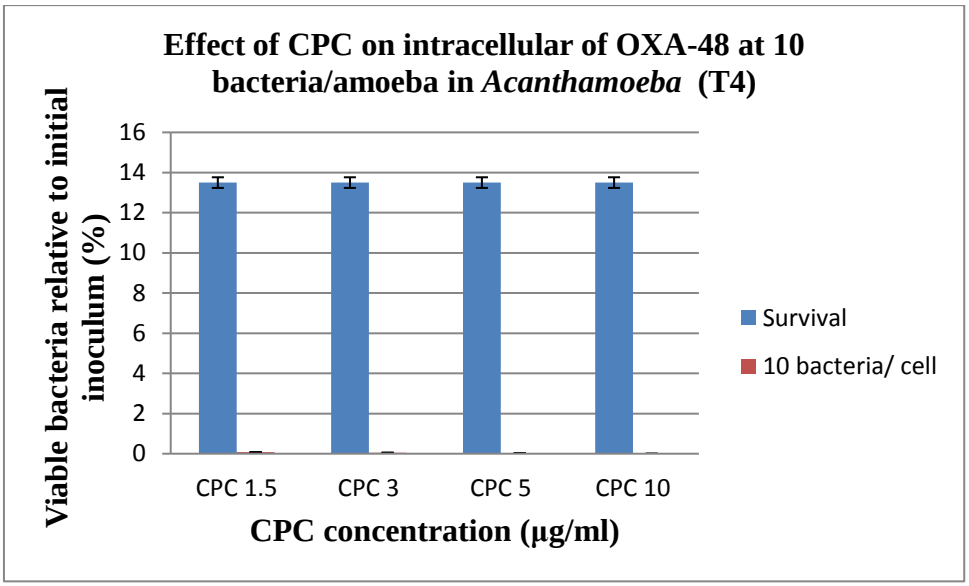

Figure 5.6 Effect of different concentrations of CPC on intra-amoebic uropathogenic bacteria (OXA-48 strain at 10 bacteria/amoeba) in *Acanthamoeba* (T4).

The percentages of bacteria relative to the initial inoculum are shown. Blue bars represent the intracellular survival of OXA-48 in the absence of CPC (control). CPC 1.5 - 1.5µg/ml; CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; and CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at $P<0.05$ (paired one-tailed t-test).

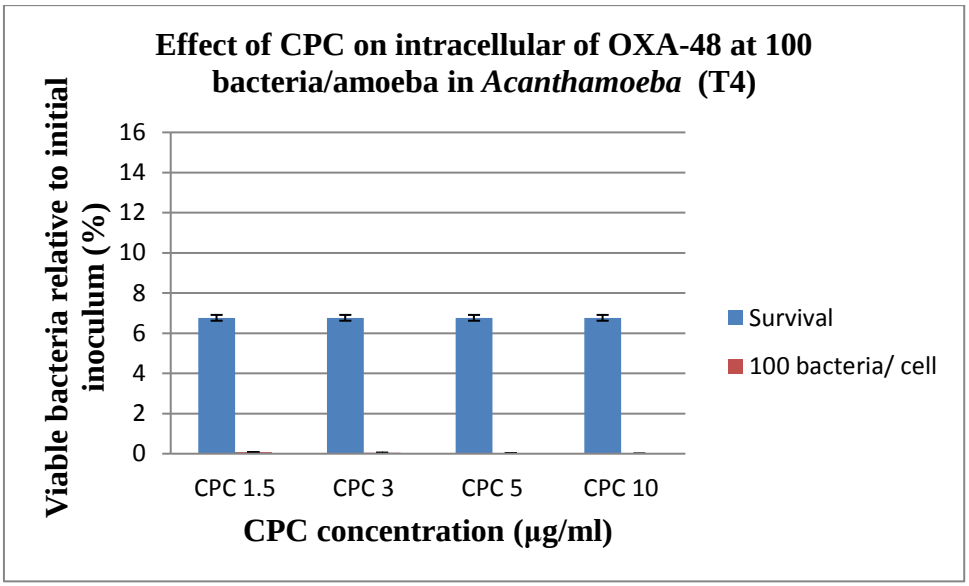

Figure 5.7 Effect of different concentrations of CPC on intra-amoebic uropathogenic bacteria (OXA-48 at 100 bacteria/amoeba) in *Acanthamoeba* (T4).

The percentages of bacteria relative to the initial inoculum are shown. Blue bars represent the intracellular survival of OXA-48 in the absence of CPC (control). CPC 1.5 - 1.5µg/ml; CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; and CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at P<0.05 (paired one-tailed t-test).

5.3.2.2 Effects of CPC on intracellular bacteria in urothelial cell line

TERT-NHUC survival assays were performed to determine the ability of CPC to kill intracellular bacteria. The experiment was conducted on the OXA-48 strain, because the OXA-48 strain was the only strain which survived and multiplied inside the urothelial cells. TERT-NHUC invaded with bacteria were incubated at 37°C for 24h. The percentage of bacteria surviving within urothelial cells were calculated as follows: number of viable bacteria recovered (CFU/ml) / number of bacteria in initial inoculum x 100 (Alsam *et al.*, 2006; Yousuf *et al.*, 2013). Figures 5.8 and 5.9 show the relationship between the four different concentrations of CPC tested against the survival of OXA-48 strain in urothelial cells, using two different infecting

doses. A paired one-tailed t-test was used to analyse the relationship between the control (OXA-48 strain survival without CPC) and OXA-48 strain at (different infecting doses) with varying concentrations of CPC (1.5, 3, 5, and 10µg /ml).

Figure 5.8 shows the comparisons between the different concentrations of CPC tested against the survival of OXA-48 at infecting doses of 10 bacteria/cell in TERT-NHUC line. Figure 5.9 shows this comparison at the higher infecting dose of 100 bacteria/cell in TERT-NHUC line. It can be seen from the data that the intracellular survival ability of OXA-48 is reduced in the presence of CPC. Furthermore, these results showed that the increased concentrations of CPC showed a decreased level of bacterial survival. No bacterial survival occurred at a concentration of 10µg/ml for either infecting dose (10 or 100 bacteria/cell). At an infecting dose of 10 bacteria/cell and a concentration of 1.5µg/ml, CPC has significantly ($P<0.05$) reduced bacterial survival to 0.014%, to 0.009% at a concentration of 3µg/ml, and to very low levels of survival (0.005%) at a concentration of 5µg/ml. Additionally, concentrations of 1.5µg/ml, 3µg/ml, and 5µg/ml of CPC gave very low levels of survival (0.012%, 0.009%, 0.029%, and 0.005% respectively) at an infecting dose of 100 bacteria per cell. (Figure 5.9). Results are significant at $P<0.05$ (paired one-tailed t-test).

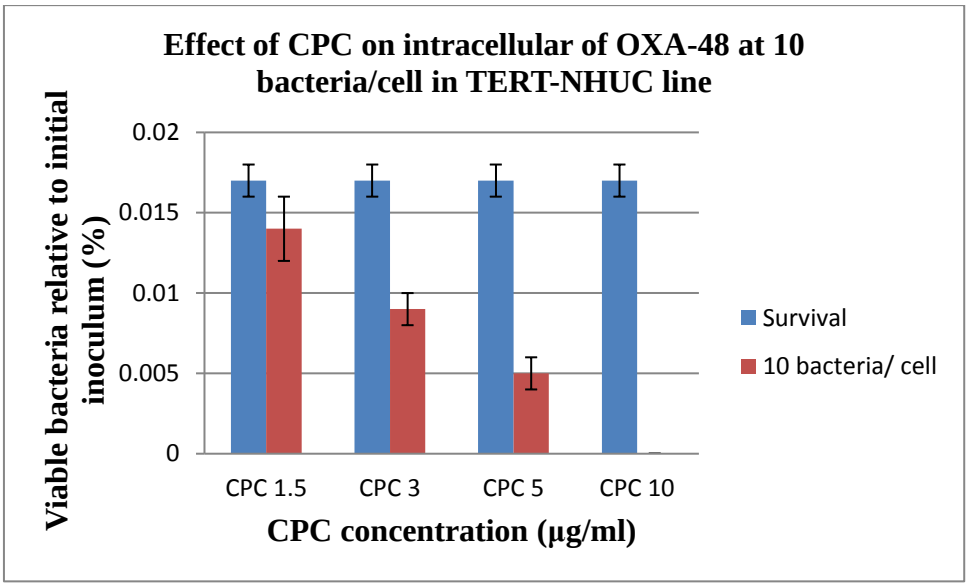

Figure 5.8 Effect of different concentrations of CPC on intracellular of uropathogens bacteria (OXA-48 at 10 bacteria/cell) in TERT-NHUC line.

The percentages of bacteria relative to the initial inoculum are shown. Blue bars represent the intracellular survival of OXA-48 in the absence of CPC (control). CPC 1.5 - 1.5µg/ml; CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; and CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at $P<0.05$ (paired one-tailed t-test).

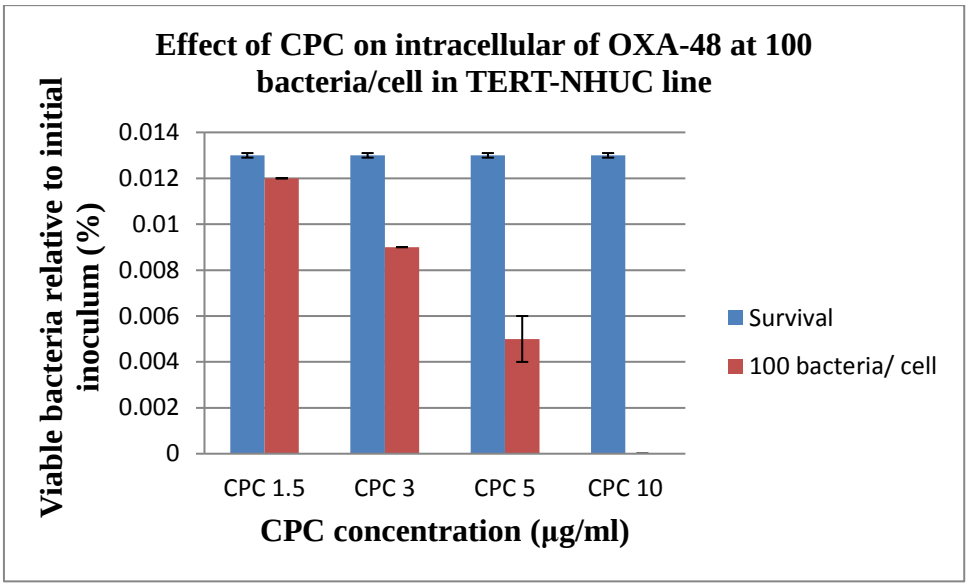

Figure 5.9 Effect of different concentrations of CPC on intracellular of uropathogens bacteria (OXA-48 at 100 bacteria/cell) in TERT-NHUC line.

The percentages of bacteria relative to the initial inoculum are shown. Blue bars represent the intracellular survival of OXA-48 in the absence of CPC (control). CPC 1.5 - 1.5µg/ml; CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; and CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at P<0.05 (paired one-tailed t-test).

5.3.3 Cytotoxicity assays by LDH

Cytotoxicity assays by LDH were performed to check the toxic effect of CPC on TRET-NHUC. In these assays, different concentrations of CPC (1.5, 3, 5 and 10µg/ml) were used, and results were compared with urothelial cells without CPC. The effect of different concentrations of CPC on urothelial cells was measured based on the amount of LDH released from cells after 24h incubation.

As evident in Figure 5.10, the results showed that the increased concentrations of CPC increased cytotoxic effect on the cell line. The minimum concentration (1.5µg/ml) of CPC has induced 18.67% cytotoxicity on TERT-NHUC line. This result is significant at P<0.05 level

(paired one-tailed t-test). The higher concentrations (3, 5, and 10µg/ml) of CPC have also induced significant cytotoxicity (P<0.05) (27.33, 36.67, and 53% respectively).

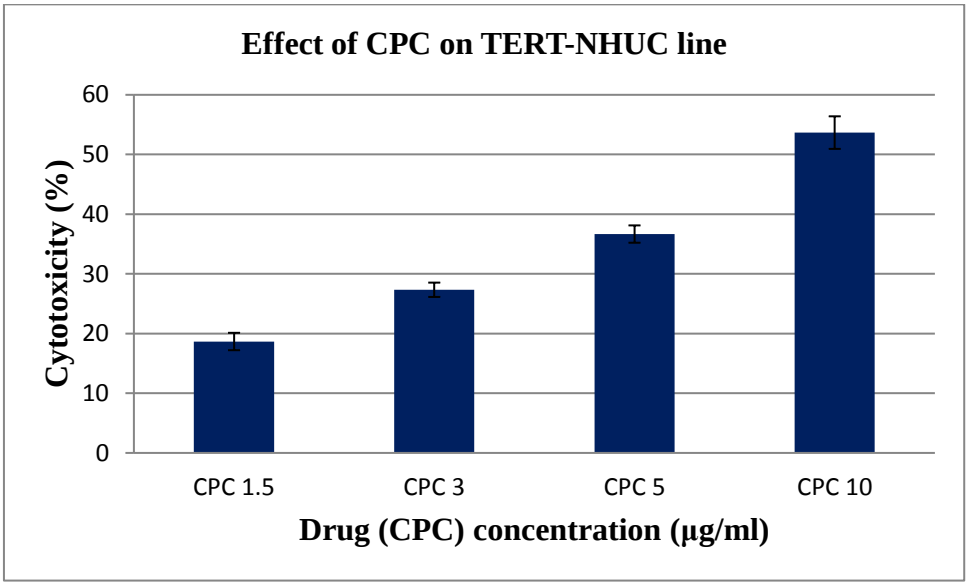

Figure 5.10 CPC induced TERT-NHUC line cytotoxicity.

Urothelial cells were incubated with different concentrations of CPC for 24h, and the cells were then assayed using a standard protocol for LDH cytotoxicity detection kit. Percentage cytotoxicity relative to control cells is shown. CPC 1.5 - 1.5µg/ml; CPC 3 - 3µg/ml; CPC 5 - 5µg/ml; CPC 10 - 10µg/ml. Results are obtained from at least three independent experiments. Error bars represent standard error. Results are significant at P<0.05 (paired one-tailed t-test).

5.4 Discussion

This study aimed to assess the antimicrobial and the intracellular antimicrobial ability of CPC and its potential use in treating UTIs.

The first part of the chapter researched at the antimicrobial effect of CPC against uropathogenic bacteria. CPC is a well-known antibacterial agent. In a previous study, CPC with a concentration of 0.05% has potent antimicrobial activity against *E. coli* (Masadeh *et al.*, 2013). Also, in a study using the agar dilution and broth dilution methods, Wu et al. (2015) found that zoonotic strains of *E. coli* related to the poultry industry were susceptible to CPC (0.1 to 1mg/ml). Although several other studies (Ransom *et al.*, 2003; Baird *et al.*, 2006) have investigated the antimicrobial impact of CPC on *E. coli*. There are no previous studies found on the effect of CPC against uropathogenic bacteria associated with UTIs. This study investigated the effects of CPC on uropathogenic bacteria, and the results (Figure 5.1 to 5.4) indicate that, at a concentration of 10μg/ml, CPC is bactericidal to all tested uropathogens bacteria over a period of 24h. The bactericidal effect of CPC is currently understood in the following terms. Electrostatic interactions between the negatively charged bacterial plasma membrane and the positively charged hydrophilic quaternary ammonium group lead to cell lysis and bacterial death (Sandt *et al.*, 2007).

The second part of the current study explored the anti-bacterial and anti-amoebic effects of CPC on the cell line and the cytotoxic effects of CPC on the cell line. Several studies have investigated the activity of biocides against free-living amoeba. However, no study has examined the impact of CPC. Alkylphosphocholine and alkylphosphohomocholine derivatives of QACs such as cetylpyridinium bromide, and benzethonium chloride were tested for their anti-amoebic properties. They found the tested QACs to have high activity against *A. lugdunensis* and *A. quina*. Even though the study did not investigate the effect of CPC on *Acanthamoeba* (T4). Their

findings are notably different from the results of the current study. CPC at concentrations of 1.5, 3, and 5µg/ml did not show any significant anti-amoebic against *Acanthamoeba* (T4) (Figure 5.5). The concentrations of 10µg/ml and higher than 10µg/ml induced encystation and decreased surface adherence.

To use for human, the United States Food and Drug Administration Plaque Subcommittee has concluded that short-term use of oral antiseptics having up to 0.1% of CPC is safe (Feres *et al.*, 2010). In a study by Muller et al. (2017), oral mouth rinses containing CPC were found to have a high *in vitro* cytotoxic effect on oral fibroblasts, murine aneuploid fibrosarcoma cells (L929), and human oral epithelial carcinoma cells (HSC-2). Also, the results of this project indicate that CPC has significant cytotoxic effects on urothelial cell lines in a dose-dependent way (Figure 5.10). In the present study, the cytotoxic investigation is based on monolayer cultures. However, the human urothelium is made of three layers including the basal, intermediate and umbrella cells. Moreover, the primary urothelial mediated response to infections and toxicity is the exfoliation of superficial cells and urothelial renewal (Nagamatsu *et al.*, 2015). Thus, it is difficult to extrapolate the *in vitro* results from the present study to the urinary tract.

The final part of the present study deals with the effects of CPC on the intracellular survival of IBCs. Many previous studies have demonstrated the bactericidal activity of different antimicrobial products on intracellular bacteria inside *Acanthamoeba* (T4), as for disinfectants such as actichlor, virkon, biocleanse & ethanol (Cardas, 2014), and iron (III) – selective hexadentate ligands of 3-hydroxypyridine-4-one chelators (Li *et al.*, 2016). The results (Figures 5.6 and 5.7) from this study showed that CPC (1.5µg/ml) has significantly increased the ability of uropathogenic bacteria (OXA-48 strain) to survive within *Acanthamoeba* (T4) compared with the control one. It may be a likely explanation that the OXA-48 strain of *E. coli* uropathogenic bacteria was able to strengthen their virulence factors whilst inside the amoebae. Several studies

have confirmed the increase in virulence of bacteria grown inside *Acanthamoeba* (Barker *et al.*, 1992; Sandström *et al.*, 2011; Van der Henst *et al.*, 2016).

Concerning the intracellular survival in urothelial cells, this study (Figure 5.10) found that OXA-48 displayed a reduced ability to survive within TERT-NHUC line in the presence of CPC in a dose-dependent manner. In a study done by Berry et al. (2009), it was demonstrated that uropathogenic *E. coli* strains have the capacity to differentially regulate virulence factors in the intracellular environment of TERT-NHUC line. Moreover, the other studies have confirmed the increase in virulence of *E. coli* found inside urothelial cells (Berry et al., 2009; Robino et al., 2014; Dikshit et al., 2015; Schreiber et al., 2017). Furthermore, many previous studies have investigated the bactericidal effect of different antimicrobial products on bacteria inside human epithelial KB epithelial cell lines (Li *et al.*, 2016).

Based on our knowledge, this is the first study to check the ability of CPC in treating UTIs. However, the clinical approval for using the CPC in oral hygiene products is a hopeful factor in its potential use for UTIs.

Quaternary ammonium compounds such as cetyltrimethylammonium bromide, cetylpyridiniumbromide, and Cetylpyridinium Chloride inactivate energy-producing enzymes, denature essential cell proteins, and disrupt the cell membrane. The quaternary ammonium compounds exhibit antiacanthamoebic activity (at <20 μM) (Siddiqui *et al.*, 2016).

In conclusion, the development of increased resistance against available antibiotics is a major global concern and is a significant threat to the successful treatment of infectious diseases. Many bacteria are developing or have already established, resistance against the currently available antibacterial agents (Fair and Tor, 2014). The indiscriminate use of various antimicrobial drugs for infectious disease treatment has resulted in the drug resistance in pathogens in recent years. Therefore, looking for new or additional methods and compounds for

treatment is urgent. By using novel compounds for the treatment of both ESBL+ve strains and *Acanthamoeba* may help many patients suffering from prolonged and recurrent of UTIs. So, this will lead to a reduction in length of hospital stay and reduce treatment costs for the patient. The current results are encouraging in our pursuit to find complementary sources of antimicrobials with novel modes of action to challenge this threat.

The next chapter deals with the investigation of the presence of *Acanthamoeba* in urine samples collected from patients suspected of UTIs by *E. coli* ESBL+ve.

CHAPTER SIX 6

6. Presence of *Acanthamoeba* in urine samples from patients with ESBL positive bacteria

6.1 Introduction

Acanthamoeba is a free-living eukaryotic opportunistic single-celled living organism. *Acanthamoeba* has unfolded everywhere the planet, and is isolated from soil, air, natural and treated water, from dental treatment units, hospitals and dialysis units, eyewash stations (De Jonckheere, 1991; Karamati *et al.*, 2016). *Acanthamoeba spp*. cuse severe infections such as keratitis and encephalitis. They feed on bacteria and act as a Trojan horse for them (Santos *et al.*, 2009). Based on 18S rRNA gene sequences, the genus *Acanthamoeba* has divided into twenty-two different genotypes (T1 – T22) (Adamska, 2016; Behera *et al.*, 2016; Taher *et al.*, 2018). Many species of *Acanthamoeba* including genotypes T1, T2, T3, T4, T5,T6, T10, T11, T12 and T15 can cause severe illnesses in humans, such as keratitis and *Acanthamoeba* granulomatous encephalitis (AGE) (Alsam *et al.*, 2003; Siddiqui and Khan, 2012). The majority of keratitis cases are caused by *Acanthamoeba* T4, also genotypes T3 and T11 associated with *Acanthamoeba* keratitis (Lorenzo-Morales *et al.*, 2015).

Bacterial infection of *Acanthamoeba* was first discovered in 1954 (Drozanski, 1952), and by 1975, bacteria were shown to take refuge within *Acanthamoeba* as endosymbionts (Proca-Ciobanu *et al.*, 1975). In 2009, Santos and his colleagues concluded that *Acanthamoeba* was found in 26% of urine samples. *Acanthamoeba* was isolated from the human stool (Zaman *et al.*, 1999). Also, previous studies showed that the pathogenic *E. coli* K1 interact with *Acanthamoeba*, and can invade and remain viable inside *Acanthamoeba* (Alsam *et al.*, 2006). Recent studies have explained that invasive *E. coli* K1 can enter human macrophages, surviving and replicating intracellularly.

The presence of *Acanthamoeba* in patients may help pathogenic bacteria in urine, protecting them against antimicrobial drugs, disinfectants, and host immunity. It is well-known that the pathogenic multi-drug resistant bacteria survive and replicate inside *Acanthamoeba* (Cardas *et al.*, 2012). In severe conditions, *Acanthamoeba* becomes cysts which act as a carrier

for spreading bacteria. It was discovered that the pathogenic bacteria become more virulent and more resistant to antibiotics if they infect an amoeba and then emerge from it (Moon *et al.*, 2011).

The organisms that cause UTIs are found in many species of gram-negative Enterobacteriaceae (Madigan *et al.*, 2009; Mazzariol *et al.*, 2017), which include *E. coli* isolated in around 75-90% of cases (Kucheria *et al.*, 2005). ESBLs are enzymes that can break down many types of antibiotics, which lead to their inactivation and can be carried on bacterial plasmids. ESBLs cause international health problems since infections caused by enzyme-producing organisms are associated with high morbidity and mortality rates and the financial burden for treatment can be costly (Dhillon and Clark, 2012). ESBLs are produced by the Enterobacteriaceae family of gram-negative bacteria, especially *E. coli* (Kim et al., 2009). The European Centre for Disease Control (CDC) and Prevention in 2011 stated that the spread of ESBL+ve *E. coli* has increased in more than half of the 28 European countries over the past four years (Beytur *et al.*, 2015).

Urinary tract infections are the most common health problem in women than men because they have a shorter urethra which is closer to the anus and vagina (Griebling, 2005; Tadesse *et al.*, 2014). Females are the most commonly affected while other high-risk groups include children and the elderly (Ali *et al.*, 2009). In addition, UTIs are diffused in women during pregnancy because of changes in the urinary tract, and hormones cause changes in the urinary tract leading to infections. Increases in urinary progestins and estrogens probably lead to a decreased resistance against bacterial entry. Urinary tract infections should be treated quickly and efficiently otherwise; this will lead to more complicated infections such as kidney diseases (Delzell and Lefevre, 2000; Tadesse *et al.*, 2014).

Re-recurrence of UTIs is most common, with 44% of patients experiencing another infection within one year and 27% of patients suffering another infection within six months.

Between one-third and one-half of the recurrent infections are caused by the same bacterial strain that initiated the infection. As a result, the recurrent infections will help the bacteria to become more resistant to antimicrobials (Hilbert, 2013).

Just a two studies (Santos *et al.*, 2009; Bradbury *et al.*, 2014) have been conducted to investigate the presence of *Acanthamoeba* in urine; this study aims to check the presence of *Acanthamoeba* in ESBL+ve urine samples collected from patients at Colchester General Hospital in 2017.

6.2 Materials and methods

All the chemicals for this study were purchased from Sigma-Aldrich unless otherwise stated.

6.2.1 Ethical approval

Application for ethical approval was required for this study. The ethical approval application had three stages. Firstly, the application was reviewed by the research and enterprise office at the University of Essex to obtain a sponsor confirmation letter. Secondly, the application was submitted online through the Integrated Research Application System, Yorkshire & The Humber –Sheffield Research Ethics Committee (Reference: 16/YH/0266). Finally, NHS Research and Development (NHS R&D) approval form and Site-Specific Information (SSI) form were completed through the IRAS website and sent with all supporting documents to Colchester General Hospital NHS Trust's Research and Development office for approval. Then the NHS R&D approval was issued.

6.2.2 Collection and processing of urine samples

A total of 74 urine samples were collected from the Microbiology Department, CHUFT over a period of one month. Equal samples from male and female patients were randomly selected and anonymised by assigning a unique identification number. About 10ml of urine from each sample was transferred into 15ml sterile tubes and centrifuged (3000 x g) for 15min. The supernatants were discarded, and the pellets were treated with 2ml of 0.25M sulphuric acid for 20min, centrifuged and washed with 3ml of PBS (3000 x g) for 5min. Sulphuric acid treatment was used to decontaminate specimens heavily contaminated with gram-negative bacteria (Public Health England, 2016b; Wenzler *et al.*, 2016).

6.2.3 Preparation of *E. coli* food source stock

To make the stock of *E. coli* suspension, *E. coli K12* was streaked into a CLED agar plate using a sterile disposable loop and incubated overnight at 37°C. Distinct single colonies were transferred into to a sterile glass bottle with 200mL of LB broth and incubated overnight at 37°C. The following day, the suspension of *E. coli* K12 was placed in a water bath at 95°C for 30min to kill the bacteria. The stock of *E. coli K12* to be used as a food source can be stored at 4°C and refreshed every two weeks.

6.2.4 *Acanthamoeba* culture

6.2.4.1 Xenic culture

One ml of the stock was added into non-nutrient agar (NNA) plates, and the culture was allowed to soak into the agar at RT for 20-30min. Plates were dried, and the pellets from the processed urine samples were inoculated onto the centre of the NNA plates. The plates were incubated at 30°C and were observed weekly for the presence of *Acanthamoeba*. A piece of agar containing trophozoites/cysts was cut from an *Acanthamoeba* positive plate. This was then placed face down onto a fresh plate of NNA that had heat-killed *E. coli*. Finally, plates were incubated at 30°C and were observed weekly for the growth of cysts or trophozoites (Khan and Paget, 2002; Khan, 2006).

6.2.4.2 Axenic culture

Pieces of agar from NNA plates which were positive for *Acanthamoeba* growth were cut by using a sterile scalpel and transferred into a T25 flask containing 7ml of PYG with antibiotics (Penicillin/Streptomycin 100U/ml and 0.1mg/ml respectively) (Hughes and Kilvington, 2001). Flasks were incubated at 30°C and checked weekly for amoebic growth. All traces of sulphuric

acid were then removed by washing the pieces with sterile deionised water; this wash was repeated three times.

To collect *Acanthamoeba*, flasks were placed on ice for 20min to detach surface adherent amoebae; the medium was collected and centrifuged at 3000 x g for 5min. The pellets were transferred to fresh T25 flasks containing PYG and antibiotics. This step was repeated once a week until the medium was free of bacterial contamination. For flasks which were contaminated with fungus were placed on ice for 20min to detach surface adherent amoebae. The medium was carefully collected and treated with 0.5M sodium hydroxide (NaOH) for 30min (Kranzer *et al.*, 2016). All traces of NaOH were then removed by washing the pieces with PBS and centrifuged at 3000 x g for 5min, and the pellets were transferred to fresh new T25 flasks containing 7ml of PYG and antibiotics.

6.2.5 Genomic analysis

6.2.5.1 DNA extraction

To extract *Acanthamoeba* DNA, they were extracted according to the manufacturer's instructions. Nonconfluent amoebae flasks approximately 20 – 30% of cysts and trophozoites were placed on ice for 20min to detach surface adherent amoebae, then the medium was collected and centrifuged at 3000 x g for 5min. DNA was extracted by Chelex resin. Two hundred µl of the Chelex solution (10% [wt/vol]) in 0.1% Triton X-100 and 10mM Tris buffer [pH 8.0]) were added to the pellets and vortexed for10s, then was centrifuged at 10000 x g for 10s and heated in a water bath at 95°C for 20min. The mixture was centrifuged at 10000 x g for the 20s again, and the supernatant (1µl) was used as a substrate for PCR (Iovieno *et al.*, 2011).

6.2.5.2 Polymerase Chain Reaction

16S rRNA primer (Table 6.1) was used for PCR amplification. PCR was performed in 50µl reaction volumes in the PCR tube as described in chapter 2 (2.2.5.1). The PCR cycling setup was different for each gene as listed in (Table 6.2).

Table 6.1 Gene and primer sequences for the *Acanthamoeba* under investigation.

Target gene	Primer (nucleotide sequence)	reference	Product length (bp)
Acanthamoeba 16S rRNA	F 5-TTATATTGACTTGTACAGGTGCT-3	(Wang *et al.*, 2002)	180 bp
	R 5-CATAATGATTTGACTTCTTCTCCT-3		

Table 6.2 The setup of PCR cycling for the *Acanthamoeba* 16S rRNA under investigation.

E. coli 16S rRNA	Temperature	Time	
		min	s
Initial Denaturation	94 °C	10	00
Denaturation	94 °C	01	00
Annealing	55 °C	00	30
Extension	72 °C	01	00
Final Extension	72 °C	10	00
Cycles	35		

6.2.5.3 Purification of PCR product

To sequence and definition the strains the PCR product was purified by using the QIAquick PCR purification kit using a microcentrifuge (QIAGEN Ltd., Crawley, UK) by following manufacturer's guidelines. In brief, 5µl of the PCR product was mixed with 25µl of

buffer PB. The mixture was further mixed with 10µl of 3M sodium acetate (pH 5.0), and when the colour of the mixture turned yellow, it was applied to a QIAquick column and centrifuged at 17900 x g for 60s. The flow-through was discarded, the product was washed with 750µl of buffer PE and centrifuged at 17900 x g for 60s. Again, the flow-through was discarded, and the QIAquick column was centrifuged at 17900 x g for another 60s. Then, the QIAquick column was placed in a clean 1.5ml microcentrifuge tube, and DNA was eluted by adding 30µl elution buffer to the centre of the QIAquick membrane. The column was allowed to stand at RT for 1min and centrifuged at 17900 x g for the 60s.

6.2.5.4 Gene sequencing

To definition the strains purified PCR products were commercially sequenced by using Sanger Sequencing Services (Eurofins Scientific, Wolverhampton, UK). Forward *Acanthamoeba* 16S rRNA (R 5-CATAATGATTTGACTTCTTCTCCT-3) primer was used to obtain the sequences.

6.2.5.5 BLAST search and phylogenetic analysis

Genotype assignment was based on sequence analysis of the 16S rRNA gene. Sequences were identified by using the nucleotide Basic Alignment Search Tool (n-BLAST). Nucleotide collection database from GenBank was used, and the program selection was optimised for highly similar sequences (megablast). Phylogenetic analysis based on the sequences obtained was performed using MEGA with default settings and configured for highest accuracy and phylogeny.fr platform (Dereeper *et al.,* 2008). MUSCLE (v3.8.31) was used to align the sequences with default settings and configured for highest accuracy. Finally, the tree was graphically represented and edited with TreeDyn (v198.3).

6.3 Results

Thirteen out of sixty-seven (17.11%) urine samples were positive for *Acanthamoeba* (Table 6.3). All samples were anonymised with a unique identification number, and details for the positive samples were collected from the Microbiology department, CHUFT. All positive samples and positive control were cloned to obtain pure isolations for axenification and further molecular investigation (Figure 6.1). Thirty-eight urine samples were collected from a male with just two urine samples (15.38%) tested positive for the amoebae, whereas, in the female, 11 urine samples (84.62%) tested positive for the amoebae out of 38 urine samples (Figure 6.2). n-BLAST revealed the identification of two different species of *Acanthamoeba* (Table 6.3). Out of the 13 positive samples, 6 were *Acanthamoeba sp.* (unclassified) and 7 were *A. castellanii* (Figure 6.3).

DNA extracted from positive samples was successfully amplified (Figure 6.4) for the partial sequence of the 16s rRNA gene (mitochondrial subunit), and sequences were commercially obtained from Eurofins Scientific, Wolverhampton, UK. Sequence alignment was performed with MUSCLE (v3.8.31) by using default settings and configured for highest accuracy. The phylogenetic tree (Figure 6.5) was reconstructed by MEGA and phylogeny.fr platform. The tree was graphically represented and edited through TreeDyn (v198.3).

The seven *A. castellanii* positive samples (454-23-5, 538-23-5, 434-23-5, 527-23-5, 254-5-6, 016-19-6, and 546-19-6) are clustered close to each other, whereas the six other samples (494-10-5, 707-10-05, 500-23-5, 551-23-5, 597-5-6, and 218-19-6) that relative to the organism *Acanthamoeba sp.*, are close together.

Table 6.3: List of urine samples from patients with ESBL+ve and tested positive for *Acanthamoeba*.

Organisms were identified using the n-BLAST search and accession numbers were extracted from GenBank at NCBI. Sample details were collected from the microbiology department, CHUFT. UIN – unique identification number, CD – clinical diagnosis.

N	UIN	Sample details		Organism	Accession number
		Sex	CD		
1	494-10-5	F	UTIs by ESBL	*Acanthamoeba sp.* JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1
2	707-10-05	F	UTIs by ESBL	*Acanthamoeba sp.* JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1
3	454-23-5	M	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	AF479550.1
4	538-23-5	F	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	AF479550.1
5	434-23-5	F	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	AF479550.1
6	500-23-5	F	UTIs by ESBL	*Acanthamoeba sp.* JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1
7	527-23-5	F	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	AF479550.1
8	551-23-5	F	UTIs by ESBL	*Acanthamoeba sp.* JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1
9	254-5-6	M	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	AF479550.1
10	597-5-6	F	UTIs by ESBL	*Acanthamoeba sp.* JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1
11	016-19-6	F	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene,	AF479550.1

				partial sequence; mitochondrial gene for mitochondrial product	
12	218-19-6	F	UTIs by ESBL	*Acanthamoeba sp.* JPH24 mitochondrial gene for 16S rRNA, partial sequence	AB795716.1
13	546-19-6	F	UTIs by ESBL	*A. castellanii* strain CDC V014 16S small subunit ribosomal RNA gene, partial sequence; mitochondrial gene for mitochondrial product	AF479550.1

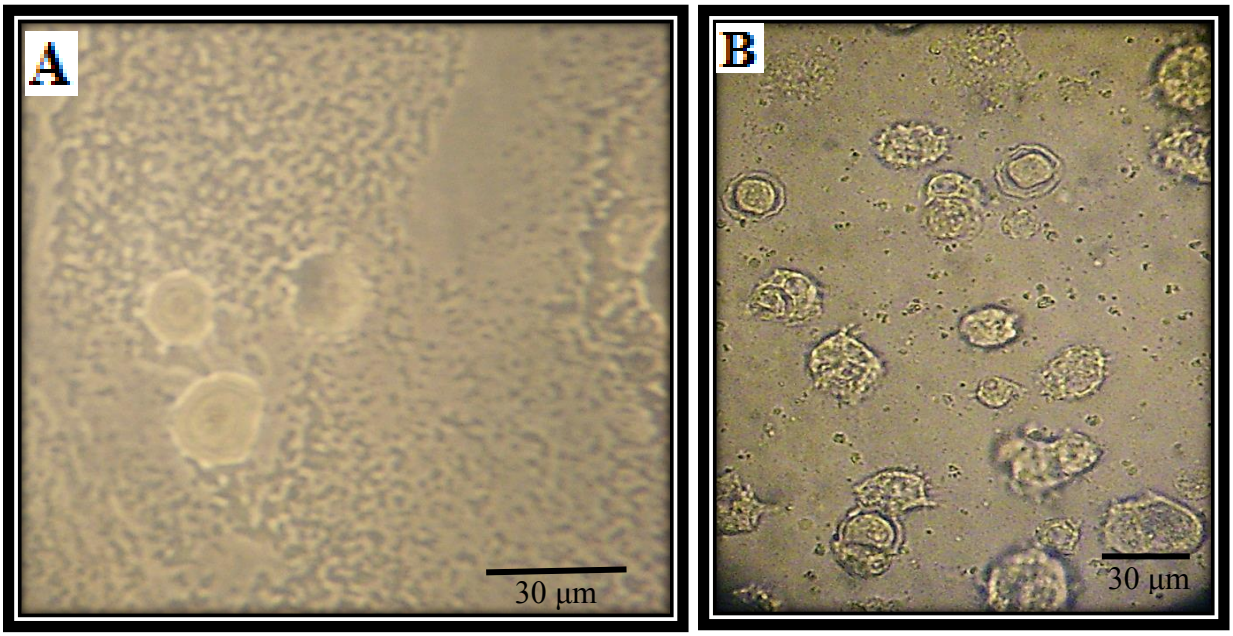

Figure 6.1: Presence of *Acanthamoeba spp.* in the positive urine samples.

Image (A) shows *Acanthamoeba,* and their feed tracks on NNA; and image (B) shows *Acanthamoeba* trophozoites and cysts on PYG. X40 objective (bars = 30 µm).

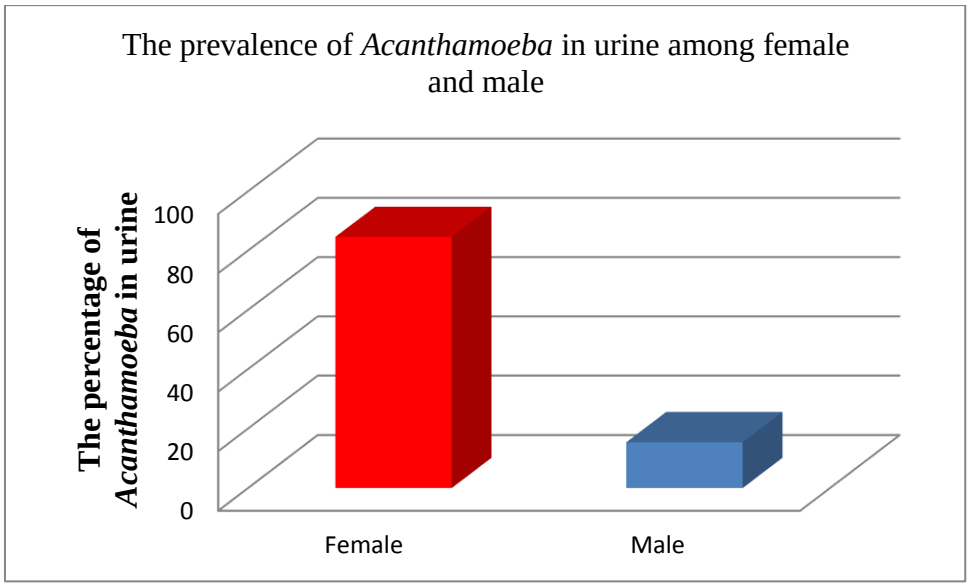

Figure 6.2: The percentage of displaying the prevalence of *Acanthamoeba* in urine among female and male.

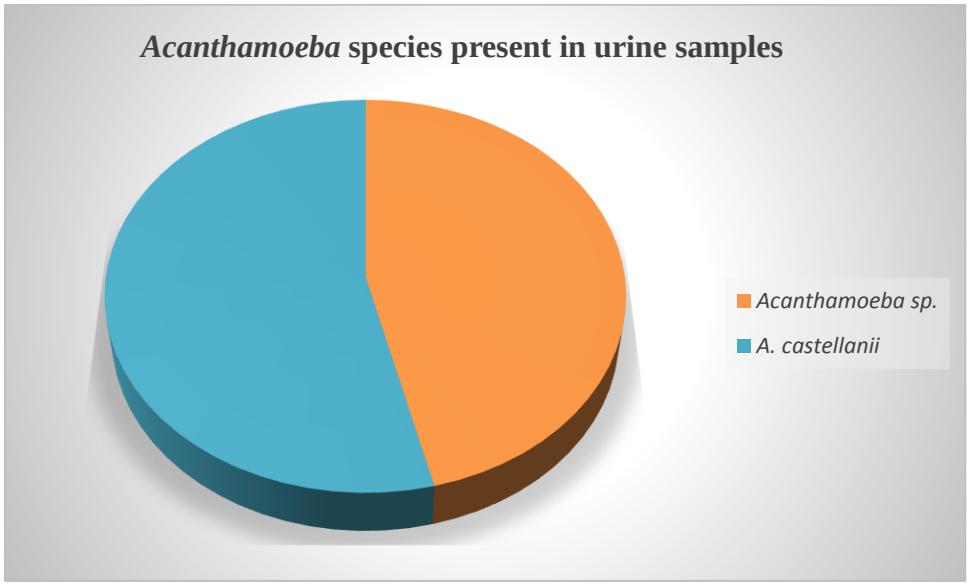

Figure 6.3: Pie chart is displaying the spread of different species in *Acanthamoeba* positive urine samples.

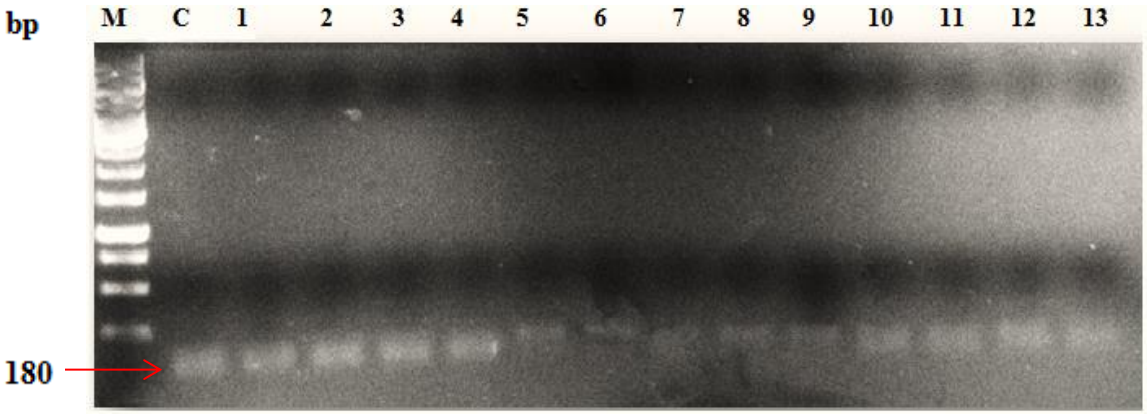

Figure 6.4: PCR products amplified using 16s rRNA primer. Bands were observed in all lanes corresponded near to the expected approximately 180 bp.

M – kbp DNA ladder, C – *Acanthamoeba* (T4 - control), 1 to 13 – positive urine samples.

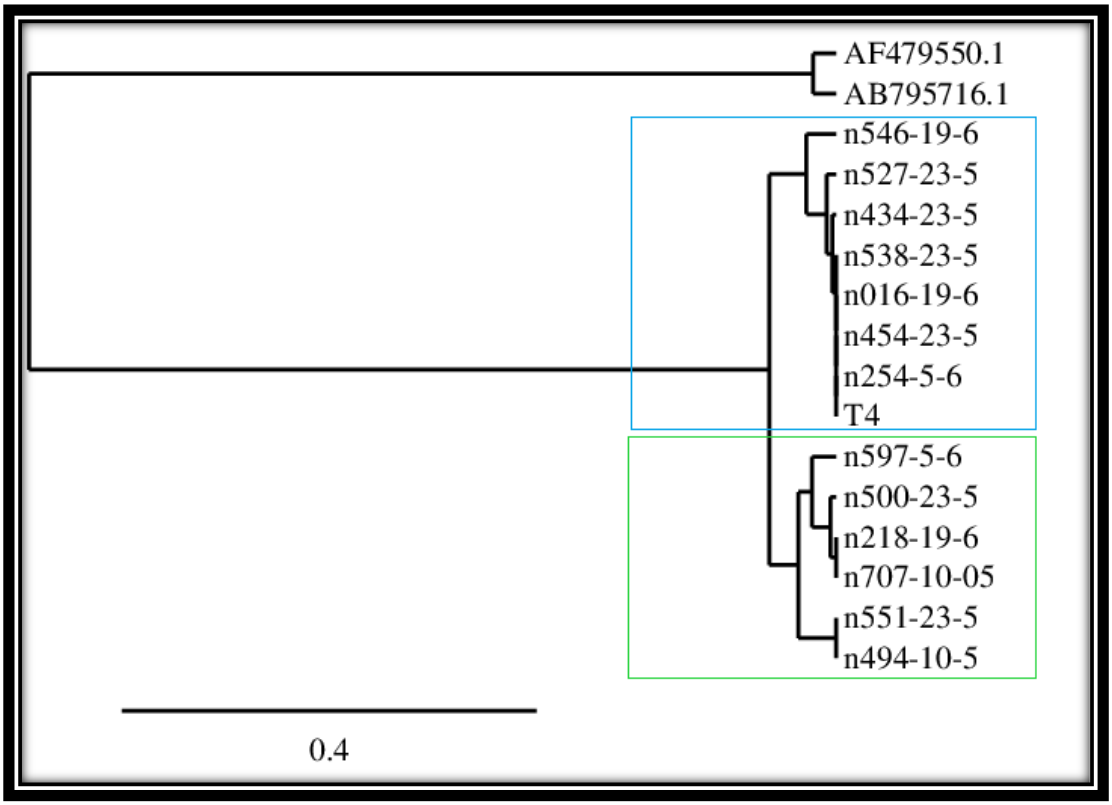

Figure 6.5: 16s rRNA phylogenetic trees for the 13 positive samples and two reference sequences.

Two reference sequences (AB795716.1 – *Acanthamoeba spp.* and AF479550.1 – *Acanthamoeba castellanii*) were used. Which is located in the blue box belong to *Acanthamoeba castellanii*, and that into the green box belong to *Acanthamoeba* spp.. The phylogenetic tree was reconstructed by the PhyML program (v3.1/3.0 aLRT) using the maximum likelihood method. The tree was graphically represented and edited with TreeDyn (v198.3).

6.4 Discussion

First worldwide study to look at the genomic characterisation of *Acanthamoeba* strains from urine samples in patients with ESBL positive bacteria. The presence of *Acanthamoeba* gives them the opportunity to be one of the microorganisms which may cause or help reoccurrence of UTIs. *Acanthamoeba* species have been isolated from both clinical and environmental sources, including waters (Siddiqui and Khan, 2012). As well, *Acanthamoeba* was isolated from the nasal of healthy people indicating that subclinical infections might be common (Bloch and Schuster, 2005; Niyyati *et al.*, 2016).

In the present study, a total of 76 samples collected from patients with ESBL positive suspected of UTIs were tested for the presence of *Acanthamoeba.* Thirteen out of sixty-seven (17.11%) urine samples were positive for *Acanthamoeba.* The results from this study were consistent with Santos study discovered from the presence of *Acanthamoeba* in the urine samples (Santos *et al.*, 2009). Bacteria that live in amoebic host contribute substantially in its capability for proliferating inside mammalian cells and cause disease. It facilitates bacteria to demonstrate improved growth in different environments where they can infect host (Sandström *et al.*, 2011). According to Segal and Shuman (1999), when bacteria live inside macrophages or amoebae, their surface characteristics get changed. Moreover, they acquire more resistance to antibiotics and more vulnerability for chemicals. Considering these reports, it can be stated that *Acanthamoeba* can harbour several different bacteria which are capable of escaping human immunity (Goy *et al.*, 2007). It has been established that the ESBL-related infections have played a significant role in worsening the issue of antibiotic resistance all across the globe (Davies and Davies, 2010; Cantón *et al.*, 2012; Winkler *et al.*, 2015). Reports have indicated transmission of ESBL-carrying infections in healthcare centres and community (Burke *et al.*, 2012; Baraniak *et al.*, 2013; Valenza *et al.*, 2014). As mentioned above, bacteria can survive

within the amoeba and in fact can become more virulent (Iovieno *et al.*, 2010; Bennett *et al.*, 2013; Paquet and Charette, 2016) and therefore difficult to treat.

Primers JDP1/JDP2 were used in several studies which target the ~450 bp fragment of *Acanthamoeba* 18S rRNA gene (Schroeder *et al.*, 2001) and primers FP16/RP16 were used that target the mitochondrial small subunit rRNA gene (Yu *et al.*, 1999; Wang *et al.*, 2002). In this study, we used 16S rRNA primers. The purpose of using the mitochondrial small subunit ribosomal RNA gene is that the sequences are shorter, more consistent in length, higher proportion of bases are alignable for sequence comparisons and they do not have any of the complications caused by multiple alleles or introns that are most common in the nuclear ribosomal RNA gene (Ledee *et al.*, 2003).

The most interesting finding in this study was eleven urine samples (84.62%) tested positive for the amoebae out of 38 urine samples were collected from female patients, whereas just two urine samples (15.38%) tested positive for the amoebae from male (Figure 6.2). This may contribute to the high occurrence of UTIs in women. Moreover, these results suggest a possible link with physiological, hormones, and anatomical changes. n-BLAST search (Table 6.3) revealed that the amoebae from six samples (46.15%) were positive for *Acanthamoeba spp.* -unclassified *Acanthamoeba*- and the other seven samples (53.85%) were positive for *A. castellanii* (Figure 6.3). *Acanthamoeba spp* are known to cause GAE and AK (Rocha-Cabrera *et al.*, 2015; Baig *et al.*, 2018). With regards to the clinical diagnosis, all patients were suspected of UTIs tending to support the hypothesis that *Acanthamoeba* plays a role in UTIs (Table 6.3).

The presence of *Acanthamoeba* in everywhere the planet special on the water and swimming pools may be consistent with the idea of contributing to increasing it is a presence in urine samples. It is known that *Acanthamoeba* produces a different of proteases that are vital for their pathogenic activity, particularly proteolysis and induction of apoptosis (Alizadeh *et al.*,

1994; Omaña-Molina *et al.*, 2013). If *Acanthamoeba* uses the haematogenous or oral route of entry to the bladder, its clinical implications can be profound. However, carrying ESBL+ve bacteria by *Acanthamoeba* may increase it is resistance and complicate treatment. In the presence of antibiotics, *Acanthamoeba* will turn to cysts and stay alive for a long time. The presence of ESBL+ve *E. coli* inside *Acanthamoeba* and it's multiplication will contribute to the increased risk of infections.

This is the first study in the UK to show the presence of *Acanthamoeba* in urine samples in patients with ESBL+ve. In the future analysis patient history, particularly their complete urine profile is required to fully explore the exact impact of *Acanthamoeba* on the urinary system, especially with ESBL bacterial infection. However, the presence of *Acanthamoeba* in urine supports our hypothesis that uropathogenic bacteria (ESBL) can establish a symbiotic relationship with the amoebae, evade host defence mechanisms, increase their virulence factors and cause rUTIs.

CHAPTER SEVEN 7

7. General discussion

7.1 Overview

The increase of UTIs caused by *E. coli* infection is associated with increased ESBL carriage and multidrug resistance and has become a major health concern worldwide. Hence, studying the ESBL producing *E. coli* bacteria is of great importance. This PhD project aimed to explore various underlying issues associated with UTIs. The prime issues investigated in this study are the formation of intracellular bacterial communities that are linked with recurrent UTIs and the possible role of *Acanthamoeba* in the pathogenesis of UTIs. Moreover, the use of CPC to treat the problem of uropathogenic antibacterial resistance was also studied.

7.1.1 Antibacterial resistance

The first antibiotic to be produced on a commercial basis was penicillin which was marketed in 1945. Flemming put forward the idea of using inhibition zones for evaluating the effectiveness of antibiotics against bacteria. Since that time, inhibition zones have turned into one of the most commonly utilised screening methods. Moreover, Flemming was also the pioneer to recommend cautious use of penicillin for preventing the development of resistance against it (Aminov, 2010). After the commercial production of penicillin, several different antibiotics were produced and efficiently used. However, there has been a gap in the discovery of new antibiotics since the 1980s when the last group of new antibiotics were introduced (WHO, 2014).

This gap was aggravated by the emergence of resistance genes owing to the intense selection pressure mediated by the unnecessary use of antibiotics (Su *et al.*, 2017). It is, therefore, the need of the hour to identify and synthesise new antimicrobial agents (WHO, 2014). One of the chief reasons behind the emergence of resistance to antibiotics is the presence of ESBL

enzymes. According to the World Health Organization (WHO), ESBL+ve *E. coli* strains are of great concern all over the world owing to their broad spectrum resistance (WHO, 2014).

It has been established that ESBL+ve gram-negative bacteria are capable of resisting the majority of β-lactams. They can also resist certain non-β-lactams like quinolones and aminoglycosides (Wragg *et al.*, 2017). Over 200 ESBLs have been characterised (Rawat and Nair, 2010a). TEM and SHV have proven to be the most common ESBLs present in gram-negative bacilli (Turner, 2005). Carbapenemases are a different group of β-lactamases that hydrolyse not only carbapenems but also other beta-lactam antibiotics. The main carbapenemase type is the class D β-lactamase OXA-48. OXA-48-producing Enterobacteriaceae have been involved in hospital outbreaks around the world (Liu *et al.*, 2016). The reason for the global dissemination of OXA-48 has been generally attributed to its being located on mobile genetic elements such as transposons, plasmids, and integrons (Schmiedel *et al.*, 2014). The spread of the gene is regularly related to the dissemination of the IncL/M-type plasmid of approximately 60 kb in size that harbours blaOXA-48 within Tn1999 transposon structures (Alousi *et al.*, 2018; Pulss *et al.*, 2018). This research has employed four primers (TEM, AmpC, CTX-M, and OXA-48) for screening the uropathogens for the presence of ESBLs.

It was found that OXA-48 strain has *OXA-48*, *TEM*, and *CTX-M* positive genes. It was found through the EUCAST antimicrobial susceptibility test that OXA-48 strain was capable of resisting all antibiotics except Ciprofloxacin used in the test. These observations indicated that the uropathogens carried ESBL resistance. Results obtained from SDS-PAGE and 2-D gel electrophoresis analysis were used as preliminary data to identify differences among cellular and extracellular protein profiles of ESBL+ve and ESBL-ve *E. coli* strains. The results for extracellular proteases showed that many spots featured in both OXA-48 and AmpC strains compared with others. Also, some spots featured in TEM strain, with many spots absent in ESBL –ve *E. coli* strain.

7.1.2 Recurrent UTIs

Pathogenesis of rUTIs involves the formation of intracellular bacterial communities (IBCs). In particular, the IBCs developed in deeper layers of the urothelium of the bladder may act as reservoirs thereby facilitating recurrence of rUTIs (Ejrnæs, 2011; Hannan *et al.*, 2012). The IBC cycle generally entails interaction, invasion of uropathogens inside the cells of the urothelium (Scott *et al.*, 2015). Antimicrobial drugs and host's immunity is evaded by the IBCs (Conover *et al.*, 2016).

Besides this, bacterial cells can also be discharged from shedding and turnover of epithelial cells leading to rUTIs (Glover *et al.*, 2014). In this research studies have revealed that all uropathogens are capable of interacting with the TERT-NHUC line, though the extent of interaction varies. It has been found that all bacterial strains used have the ability to associate with and invade *Acanthamoeba* and host cells. However, just OXA-48 was able to survive within *Acanthamoeba* and urothelial cells. Moreover, OXA-48 can remain and multiply inside *Acanthamoeba* within a harsh environment such as an environment with an antibiotic, where they can turn to cysts that may help transmit bacterial pathogens to susceptible hosts and also to cause reinfection. This research also found that OXA-48 strain demonstrates the highest potential in terms of endurance and development of IBCs inside urothelium. This recent observation is quite intriguing as a majority of reports indicate the strong potential of OXA-48 strain and UPEC for developing IBCs in comparison to other uropathogens (Garofalo *et al.*, 2007; Rosen *et al.*, 2007; Berry *et al.*, 2009; Robino *et al.*, 2013; Candan *et al.*, 2017).

Uropathogens like *E. coli* produce certain toxins so that they can cause cytotoxicity to urothelium. These include HlyA and CNF-1 by *E. coli* (Bien *et al.*, 2012; Flores-Mireles *et al.*, 2015). This research has found through cytotoxicity assays that considerable cytotoxicity to TERT-NHUC line was caused by all uropathogens. As per the findings of the flow cytometry assays, apoptosis and necrosis to urothelium by OXA-48 strain was of the highest degree. It has

already been established that apoptosis and necrosis are mainly caused by direct toxicity to a cell instead of a transition from the apoptotic pathway (Poon *et al.*, 2010). In the clinical context, there can be serious effects of this process as necrosis generally involves inflammation of nearby cells (Kumar *et al.*, 2015). When the intracellular bacterial formation is compared with cytotoxicity, it is found that the extent of late apoptosis and/or secondary necrosis brought about on urothelium is correlated with the degree of IBCs developed. It implies that the uropathogens cause considerable cytotoxicity in the urothelium besides building a protective reservoir for their survival.

Prevention strategies for rUTIs consider the intracellular bacterial communities. IBCs have been reported to resist antibacterial drugs by mediating phenotypic modifications in the inert intracellular areas. Resistance is also noticed in the case of antibiotic therapy with a low dose and prolonged use (Dason *et al.*, 2011; Barber *et al.*, 2013; Lüthje and Brauner, 2016). Considering this resistance, the development of alternative treatment methods has become unavoidable. Researchers have reported limited but quite hopeful results for using lactobacillus preparations (Foxman and Buxton, 2013) and D-mannose (Beerepoot and Geerlings, 2016) for preventing rUTIs.

7.1.3 Possible role of *Acanthamoeba* in UTIs

Being an opportunistic protist, *Acanthamoeba* is a free-living amoeba (Karaś *et al.*, 2018). It is found in trophozoite and cyst form. Trophozoite form of *Acanthamoeba* is capable of feeding on different bacterial cells by phagocytising them (Alsam *et al.*, 2005; Kuburich *et al.*, 2016; Maisonneuve *et al.*, 2017). This organism has recently attracted researchers owing to its endosymbiotic association with bacteria (Müller *et al.*, 2016). Several different pathogenic

bacteria have been found to live and grow inside *Acanthamoeba.* These include *E. coli* (Alsam *et al.*, 2006; Yousuf *et al.*, 2014), *V. cholera* (Abd *et al.*, 2004) and *F. tularensis* (Abd *et al.*, 2003).

Although *Acanthamoeba* had been found in healthy individuals or individuals with non-*Acanthamoeba* infections like pneumonia, this organism is involved in two rare but severe infections. These are AK and GAE. Antibodies against *Acanthamoeba* have also been detected in healthy individuals. It implies that this organism may survive in the human body without causing any infection. This raises the question if they have any role in the human physiology or if they can affect the pathology or recurrence of any infection like UTIs.

Role of *Acanthamoeba* in UTIs has been proposed by numerous researchers. There are certain considerations in this regard. First, this free-living organism is found in several different environments that include the environment of a hospital (Fukumoto *et al.*, 2016), public water systems (Jeong and Yu, 2005; Sente *et al.*, 2016), natural water bodies (J. Lorenzo-Morales *et al.*, 2005; Nuprasert *et al.*, 2010) and bottled water (Visvesvara *et al.*, 2007; Maschio *et al.*, 2015). Consequently, the probability of entry of this amoeba in the human body is very high. Secondly, feeding on bacteria by *Acanthamoeba* is an established fact (Alsam *et al.*, 2006; Maisonneuve *et al.*, 2017) and these bacteria can be those included in the normal flora of humans like bacteria present in the bladder and intestinal tract (Lewis *et al.*, 2013). Still, as discussed earlier, bacteria may not only live inside *Acanthamoeba* but may also gain more virulence (Iovieno *et al.*, 2010; Guimaraes *et al.*, 2016; Gomes *et al.*, 2018). For that reason, infections involving these pathogens are quite complicated. Thirdly, researchers have reported the isolation of *Acanthamoeba* from urine samples which were taken from patients suffering from severe diseases (Santos *et al.*, 2009). In the light of these facts, it can be proposed that *Acanthamoeba* can be essentially involved in the pathogenesis of UTIs, specifically in recurrence of infections.

Interaction assays were conducted to study the nature of the association between *Acanthamoeba* and uropathogens. As per the results of interaction assays, all tested uropathogens were capable of demonstrating interaction with *Acanthamoeba* (T4). More importantly, OXA-48 strain was capable of evading phagocytosis and continuing to survive inside amoebae. In other words, these pathogens are able to exploit amoeba for their protection against antibacterial mechanisms.

An important finding made during this research was the detection of *Acanthamoeba* in urine from patients with ESBL+ve suspected of UTIs. For this purpose, 76 urine samples collected from UTI patients were taken from the CHUFT. *Acanthamoeba* was detected in 13 (17.11%) urine samples through NNA culture method and PCR technique. When the gene for the small subunit of mitochondria (16S rRNA) was sequenced, six (7.89%) urine samples were found to contain *Acanthamoeba* (unclassified species), and seven (9.21%) samples were found to contain *Acanthamoeba castellanii*. These results are not only in agreement with the findings of previous research (Santos *et al.*, 2009) but also provide evidence for supporting the hypothesised role of *Acanthamoeba* in UTIs. It is for the first time that *Acanthamoeba* has been isolated from urine samples from patients who have ESBL+ve suspected UTIs in the UK.

Considering the abovementioned observations, it can be stated that *Acanthamoeba* might have a role in the pathogenesis of UTIs. This research has therefore revealed several facts about the association between uropathogens and *Acanthamoeba*.

7.1.4 Alternative efficient treatment strategy

Bacterial cells may resist the action of antibacterial agents in several ways which can be genetic or mechanistic. They may form IBCs and live inside *Acanthamoeba*. In this way, these pathogens become even more virulent. A useful strategy for addressing the issue of antibiotic

resistance is the utilisation of non-antibiotic agents for the treatment of infections. In this connection, the utilisation of QACs has been reported by several studies (Jiao *et al.*, 2017). An important QAC is the CPC which demonstrates strong antibacterial action against a wide range of bacteria (Hurt *et al.*, 2016).

However, the antibacterial potential of CPC has only been researched in terms of oral care (Hwang *et al.*, 2013) and processing of poultry (Arritt *et al.*, 2002; Waldroup *et al.*, 2010). This is the first time that CPC has been studied for its activity against uropathogens which are implicated in UTIs. It has been found through antimicrobial assays that CPC is capable of destroying more than 83% of uropathogens in concentrations as low as 3μg/ml. Even though it has been observed that CPC demonstrates cytotoxicity to the cells of urothelium; very low concentrations are bactericidal. Considering the fact that utilisation of CPC has been approved for oral care, small concentrations of this product can be investigated for utilisation in the treatment of UTIs.

It has been found during this research that CPC is needed in higher concentrations for diminishing intracellular bacteria in TERT-NHUC line and *Acanthamoeba* (T4). Still, QACs can be structurally altered for improving the action against bacteria. In particular, the antimicrobial activity of QACs can be improved by altering the alkyl chain lengths. This can be done by using chlorine, methyl or ethyl groups for substituting the hydrogen of the aromatic ring (Melin *et al.*, 2014; Morkaew *et al.*, 2017). As mentioned earlier, QACs containing longer chains are better in antimicrobial action as compared to those with shorter chains (Ioannou *et al.*, 2007). Hence, new QACs can be developed with altered alkyl chain length to specifically target IBCs and hence treat UTIs. One more important finding of the bacteria-amoeba intracellular survival assays is that growth of OXA-48 was found to be increased within *Acanthamoeba* in the presence of 3μg/ml of CPC which indicates a potential increment in the virulence of the uropathogen.

7.2 Conclusions

In conclusion, this research has shown that:

1. ESBL+ve strains were able to associate and invade into the *Acanthamoeba* and TRET- NHUC line.

2. OXA-48 is highly toxic, more virulent and resistant than the other ESBL strains. Moreover, OXA-48 was the only strain that survived and multiplied inside *Acanthamoeba* and TRET-NHUC line.

3. Uropathogenic cytotoxicity on urothelial cells is dependent on the number of Intracellular bacteria, and uropathogens complete their IBC pathogenic cycle in a relatively short window of 24h.

4. *Acanthamoeba* may play a role in the pathogenesis of UTIs specially rUTIs. Moreover, uropathogens have exhibited an endosymbiotic relationship with *Acanthamoeba*.

5. CPC can be potentially used in treating UTIs.

7.3 Recommendations for future work

The present study confirms previous findings related to intracellular bacteria, and their intracellular growth rate is one of the major causes of cell cytotoxicity and adds to the growing body of literature. The study also contributes additional evidence that suggests a possible role of *Acanthamoeba* and ESBL producing strains of *E. coli* in recurrent urinary tract infections. Furthermore, this research will serve as a base for future studies on the use of QACs in treating UTIs. It is recommended that further research be undertaken in the following areas:

1. Further investigations of the virulence factors most responsible for cytotoxicity could be worth studying. To assess that, a few cell types derived from the healthy human bladder or a skin tissue model could be used. In addition, different bacterial strains lacking one or more of the virulence factors could be used on the above-mentioned cells. Many techniques would be helpful to assess that: at proteins level, such as flow cytometry, western blot using antibodies; and at the gene level, such as PCR, sequencing and bioinformatics.

2. Time and financial constraints prevented further analysis (sequencing) of the spots identified from the 2D Gel analysis of the protein repertoire of the strains under study. Therefore, sequencing of selected spots (such as the potential K channel blocker) will further shed light on the differences between the strains (TEM, AmpC, and OXA-48).

3. Use whole genome sequencing to confirm the VF, phylogrouping, and sequence typing data.

4. The current investigation on uropathogen induced cytotoxicity was limited in terms of time course experiments. It would be useful to explore the cytotoxic effects of uropathogens at different incubation periods.

5. Further investigations on the ability of different strains of ESBL producer uropathogens to interact with *Acanthamoeba spp*, especially with non-pathogenic amoeba-like *Acanthamoeba* T7. Isolated from urine samples will shed more light on the exact nature of their relationship in the urinary tract. Future *Acanthamoeba* isolation studies should also evaluate the complete urine profile and clinical history of patients to fully explore health implications related to the presence of the amoeba in urine.

6. Both the results obtained and the limitations identified in this study suggest that future work in the area of ESBLs would be beneficial. As this study has highlighted, the epidemiological profile of ESBL producing bacteria is continuously evolving, and epidemiological data should be

collected yearly in an attempt to predict antimicrobial resistance patterns and identify potential shifts in resistant determinant expression and *in vivo*.

7. Finally, further investigation and experimentation into the use of CPC or other potent antimicrobial QACs such as Alkylphosphocholine, alkylphosphohomocholine, cetylpyridinium bromide, and benzethonium chloride to treat urinary tract infection especially those caused by ESBL+ve *E. coli* and *Acanthamoeba spp*.

APPENDICES

Appendix one: Microorganisms and cell line

1 Bacteria

1.1 Bacterial strains

E. coli (three strains of ESBL+ve (TEM, AmpC, and OXA-48) and one strain of ESBL-ve), isolated from patients diagnosed with UTIs were kindly provided by Dr Tony Elston, CHUFT.

1.2 Storage of bacterial cultures

By using a sterile disposal loop, about 20 isolated colonies of bacteria were picked up of the agar plate and inoculated in a 10ml of bacterial freezing medium (70% Luria-Bertani broth and 30% glycerol v/v). Then, the mixture of freezing medium with bacteria was divided into cryotubes final volume of each tube was 1ml and stored in a -20°C freezer.

1.3 Thawing and re-culturing bacteria

To re-culture bacteria, a loopful of the frozen cryotube was streaked onto a fresh agar plate and incubated at 37°C in air incubator overnight. After the incubation, the plate was carefully examined if the colonies had similar morphologies and stored at 4°C for up to a week.

2 Acanthamoeba

2.1 Acanthamoeba strain

Acanthamoeba T4 (ATCC 30234) isolated from a patient diagnosed with *Acanthamoeba* keratitis used in this study was kindly provided by Dr Sutherland Maciver, University of Edinburgh.

2.2 *Acanthamoeba* storage

Acanthamoeba can be stored as axenic cultures at -80°C. Briefly, growing amoebae were re-suspended at a density of 1-5 x 10^6 cells per ml in the freezing medium (90 % PYG medium and 10 % DMSO v/v) placed into a cryotube. The cryotube was quickly placed in a box with ice and transferred to -20 °C for 60 min followed by their storage at -80°C freezer.

2.3 Preparation of *Acanthamoeba* culture

Thawing and recovery of *Acanthamoeba* from -80°C freezer was done quickly. The cryotubes were placed immediately in the warmer incubator maintained at 37°C for 3min. *Acanthamoeba* was transferred into a 15ml tube with warmed PYG and centrifuged at 3000 x g for 5min. Next, supernatants were discarded, pellets were re-suspended in growth medium (PYG) and transferred into a T25 culture flask. Flasks were incubated at 30°C in air incubator till they become confluent.

2.4 Counting *Acanthamoeba*

The number of *Acanthamoeba* was determined by using the haemocytometer. After the haemocytometer and the coverslip were cleaned using 70% ethanol, 7µl of cell suspension were placed on a haemocytometer, and the average number of *Acanthamoeba* in 1mL was calculated. The *Acanthamoeba* were diluted to the required number using appropriate medium and plated in flasks or plates as required by experimental design.

2.5 Determination of the viability of *Acanthamoeba*

To determine the viability of *Acanthamoeba* trophozoites, the trypan blue exclusion test was used. *Acanthamoeba* was incubated at room temperature with 0.2 % trypan blue for 20min and counted by using the haemocytometer.

3 TERT-NHUC line

3.1 Cell line

TERT-NHUC line was kindly provided by Prof. Knowles, Cancer Research UK Clinical Centre, St. James's University Hospital, Leeds.

3.2 TERT-NHUC line storage

Cells were stored as axenic cultures at -80°C. Briefly, growing cells were re-suspended at a density of 1-5x10^6cells/ml in the freezing medium (90% FBS, and 10% DMSO v/v) and placed in a cryotube. The protocol was carried out gradually a cryotube on the ice, then -20°C for 60min followed by their storage at -80°C freezer.

3.3 Preparation of TERT-NHUC line culture

Thawing and recovery of stored cells from -80°C freezer were done quickly. The cryotubes were placed immediately in the warmer incubator maintained at 37°C for 3min. Cells were transferred into a 15ml tube with warmed medium KGM-2 and centrifuged at 1000 x g for 5min. Next, supernatants were discarded, pellets were re-suspended in growth medium KGM-2 and transferred into a T25 tissue culture flasks. Flasks were incubated at 37°C in 5% CO_2 and 95% humidity until the cells were confluent.

Appendix two: Media

2.1 Reagents for AUM

Ammonium chloride	Sigma A9434
Calcium chloride dihydrate Sigma	Sigma C3306
Citric acid	Sigma 251275
Creatinine	Sigma C4255
Di-potassium hydrogen phosphate	Sigma
Iron II sulphate heptahydrate	Sigma F8633
Lactic acid	Sigma
Magnesium sulphate heptahydrate	Sigma M2773
Peptone L37	Oxoid LP0034
Potassium dihydrogen phosphate	Sigma P5655
Sodium bicarbonate	Sigma S5761
Sodium chloride	Sigma S7653
Sodium sulphate heptahydrate	Sigma 71969
Urea	Sigma U5378
Uric acid	Sigma U2625
Yeast extract	Sigma Y1625

2.2 Reagents for TERT-NHUC line

Accutase	Sigma A6964
Cell lytic M	Sigma C2978
Keratinocyte growth medium 2 ready-to-use	PromoCell C-20011

2.3 Final supplement concentrations of KGM-2 media

Bovine Pituitary Extract	0.004ml/ml
$CaCl_2$	0.06mM
Epidermal Growth Factor recombinant human	0.125ng/ml

Epinephrine	0.39µg/ml
Hydrocortisone	0.33µg/ml
Insulin recombinant human	5µg/ml
Transferrin, holo human	10µg/ml

2.4 Growth medium (w/v)

2.4.1 PYG: Protease peptone (1.25g), yeast extract (1.25g), glucose (3.0g), dw (up to 1000ml) prior to autoclaving at 121°C for 15min.

2.4.2 LB broth: 10g tryptone, 5g yeast extract and 10g NaCl were placed in water to a final volume of 1000ml, was adjusted the pH to 7.5 before autoclaving at 121°C for 15min.

2.5 Agar (w/v)

2.5.1 CLED (Sigma 55420): 36.15g of CLED agar dissolved in 1000ml distilled water prior to autoclaving at 121°C for 15min.

2.5.2 Non-nutrient: 30g bacteriological agar dissolved into 1000ml Ringers solution prior to autoclaving at 121°C for 15min.

2.5.3 Mueller Hinton agar (Sigma 70191): 38g of medium dissolved in 1000ml distilled water prior to autoclaving at 121°C for 15min.

Appendix three: PCR and gel electrophoresis

3.1 PCR

3.1.1 Primer preparation

The tubes were spin at top speed for 10min to ensure that there are no lyophilised primers stuck to the cap. To make a typical 100mM 100X stock concentration of primers, the primers were dissolved in a volume of sterile distilled water that is 10X the amount of nmoles in the tube, using µl of water. This value is printed on the side of the tube. All sets of primers were stored at -20°C. Typically 0.1-1.0µM final concentration was used in a PCR mix.

3.1.2 Reagents for PCR and gel electrophoresis

Agarose	Fisher Scientific 10776644
dNTP Mix	Thermo Scientific TM R0241
GeneRuler 1 kb DNA Ladder	Thermo Scientific TM SM0311
SafeView Nucleic Acid Stain	NBS-Biologicals NBS-SV1
Taq DNA Polymerase	Thermo Scientific TM EP0401

3.1.3 TAE buffer (50X)

Tris base w/v	242g
Glacial Acetic Acid v/v	57.1ml
0.5M EDTA pH8 v/v	100ml
Distilled water	Make-up to 1000ml

3.1.4 Agarose gel (1%)

40g of agarose were dissolved in 400ml 1X TAE buffer at 50°C then 1ml of safeview was added before pouring in the electrophoresis tray.

3.1.5 Chelex solution for DNA extraction

10% (w/v) chelex were dissolved in 0.1% Triton X-100 and 10mM Tris buffer (pH 8.0).

3.2 gel electrophoresis

3.2.1 SDS-PAGE buffers

1.5M Triethylamine hydrochloride (Tris) pH 7.4	18.1g dissolved in 100ml distilled water, adjust pH to 7.4 using HCl.
0.5M Tris pH 6.8	6.057g in 100ml distilled water, adjusted pH to 6.8 using HCl.
10% (w/v) SDS	10g SDS in 100ml distilled water.
Running buffer for SDS-PAGE stock solution 5X	Tris 15g, glycine 72g, SDS 5g, make up to 1000ml with distilled water. Adjust pH to 8.3 with HCl. Dilute to 1X with distilled water prior to use.
Sample buffer 2X	0.06M Tris, 2.5% glycerol (Sigma G5516), 0.5% SDS, 1.25% 2- mercaptoethanol (Sigma M6250) and bromophenol blue 0.001% (Sigma B0126) (pH=6.8).
Coomassie brilliant blue stain	0.5% w/v Coomassie brilliant blue, 40% methanol v/v, 10% acetic acid v/v, 50% distilled water v/v.
De-stain	40% methanol v/v, 10% acetic acid v/v, 50% distilled water v/v.
Ammonium persulphate	4% w/v dissolved in distilled water

3.2.2 Running gel (10% SDS)

Distilled water	4.2ml
Bis/acrylamide solution	3.3ml
SDS 10%	100µl
Tris 1.5M pH	7.4 2ml
10% Ammonium persulphate	400µl
TEMED solution	6µl

3.2.3 Stacking gel (5%)

Distilled water	4.3ml
Bis/acrylamide solution	0.8ml
Tris 0.5M, pH 6.8	1.88ml
SDS 10%	60µl
10% Ammonium persulphate	750µl
TEMED solution	3µl

3.2.4 SDS-PAGE protocol

The Bio-Rads mini-protein electrophoresis unit 67S12533, Bio-Rad, Hemel Hempstead, UK was assembled according to the manufacturers' instructions. The running gel reagents were mixed and loaded into the sealed gel caster overlaid with 70% ethanol to exclude oxygen; this is a vertical SDS-PAGE system that cast gels of 0.75mm thickness. After polymerisation, the ethanol was decanted, and the stacking gel was mixed and layered on top of the running gel. The comb holds up to 20min were inserted into the stacking gel and polymerisation allowed occurring. The combs were then removed, and gels were assembled into the electrophoresis tank Bio-Rad 165-2949 and the tank filled with 1 X running buffer as mentioned above. Samples

mixed with sample buffer 1:1 were added to the wells and electrophoresed for 2h, 115V. Gels were then stained.

3.2.5 Buffer Recipes for 2D gel

3.2.5.1 Rehydration solution preparation

Just prior to use, the appropriate volume of stock solution was slowly thawed. The appropriate amount of IPG Buffer was added, if it is not already included in the rehydration stock solution. Sample was added. The rehydration stock can be diluted no more than 1/8 by sample addition (e.g. 62µl of the sample for each 0.5mL rehydration stock). The amount of protein that can be added is dependent upon the length of the strip, the pH range and the detection method to be used. Up to 1mg can be added to a wide pH range 17cm strip.

Note: DTT and the sample must be added fresh, just prior to use.

3.2.5.2 SDS equilibration buffer

Component	Final concentration	Amount
1.5M Tris-Cl, pH8.8	50mM	6.7ml
Urea (FW 60.06)	6M	72.07g
Glycerol (87% v/v)	30% (v/v)	69ml
SDS (FW 288.38)	2% (w/v)	4.0g
Bromophenol blue	trace	(a few grains)
Distilled water		to 200ml

(50mM Tris-Cl pH 8.8, 6M urea, 30% glycerol, 2% SDS, bromophenol blue, 200ml)

Store in 40mL aliquots at –20 °C.

3.2.6 Sample buffer

4ml of distilled water, 1ml of 0.5M Tris (v/v), 0.8ml of glycerol (v/v), 1.6ml of 10% SDS (v/v), and 0.05% bromophenol blue (w/v in distilled water) were mixed together.

3.2.7 Lysis buffer for protein extraction

7M urea, 2M thiourea, 4% 3-[3-cholamidopropyl dimethylammonio]-1- propanesulfonate (CHAPS), 15mM pH 8.5 TRIS.

3.2.8 Protocol for Bradford protein determination assay

Bradford assay is a simple and accurate procedure for determining the concentration of protein in solution that involves the binding of Coomassie Brilliant Blue G-250 dye to proteins. As more protein present as more Coomassie binds. The assay is colourimetric. As the protein concentration increases, the colour of the test samples becomes darker.

The Bradford assay was performed in a 96-well plate. The protein concentration from the test sample was determined by comparison to 5 different concentrations of the protein standards (bovine albumin serum). The standard concentrations used were: 2mg BAS/ml, 1mg BAS/ml, 0.5mg BAS/ml, 0.25mg BAS/ml and 0.125mg BAS/ml:

- 2mg BSA dissolved in 1ml of PBS, to be 2mg/ml
- 50µl from 2mg/ml of BSA were diluted into 50µl of PBS, to be 1mg/ml
- 50µl from 1mg/ml of BSA were diluted into 50µl of PBS, to be 0.5mg/ml
- 50µl from 0.5mg/ml of BSA were diluted into 50µl of PBS, to be 0.25mg/ml
- 50µl from 0.25mg/ml of BSA were diluted into 50µl of PBS, to be 0.125mg/ml

There were standard wells, unknown sample wells and blank well:

Standard 1: 200µl Bradford reagent (BR) (Sigma B6916) + 5µl of 2mg BSA/ml

Standard 2: 200µl BR + 5µl of 1mg BSA/ml

Standard 3: 200µl BR + 5µl of 0.5mg BSA/ml

Standard 4: 200µl BR + 5µl of 0.25mg BSA/ml

Standard 5: 200µl BR + 5µl of 0/125mg BSA/ml

Unknown sample: 200µl BR + 5µl of sample

Blank: 200µl BR + 5µl PBS

The samples were mixed using a microplate mixer and incubated at room temperature for at least 5min. The spectrophotometer was set to 595nm, and the absorbance of standards, blank and unknown sample were measured. A standard curve was created by plotting the 595nm values (y-axis) versus their concentrations in µg/ml (x-axis). The unknown sample was determined by using the standard curve.

Appendix four: Antibiotics, antimicrobial agents, and the other reagents used

4.1 Antibiotics preparation

Meropenem: stock solution: 1mg/mL (w/v) dissolved in distilled water

4.2 Antibiotics and antimicrobial agents

Antibiotic discs	Oxoid
Cetylypyridinium chloride	Sigma C0732
Meropenem trihydrate	Sigma M2574
Penicillin-Streptomycin	Sigma P4333

4.3 Phosphate buffered saline (PBS tablets)

One tablet dissolved in 200ml deionized water giving 0.01M phosphate buffer containing potassium chloride (0.0027M) and sodium chloride (0.137M). The sample was autoclaved at 121°C for 15min. Adjust pH 7.4 at 25°C.

4.4 Disinfections used in the laboratory:

1% virkon (w/v): 1mg of virkon powder in 100ml PBS

70% ethanol (v/v): 70ml of concentrated ethanol in 100ml H_2O

4.5 Reagents for flow cytometry

Annexin V Binding Buffer	BioLegend 422201
Annexin V-FITC	BioLegend 640906
Cell staining buffer	BioLegend 420201
Zombie NIR	BioLegend 423106

4.6 Other reagents used

Acetic acid	Fisher Scientific 10375020
Acetonitrile	Sigma 271004
Ammonium persulphate	Sigma A3678
Bis/acrylamide solution for electrophoresis	Sigma A3574
Bovine serum albumin	Sigma A2153
CelLytic MT Cell Lysis Reagent	Sigma C3228-500 ml
CHAPS	Sigma C9426
Chelex	Sigma C7901
Chloroform	VWR international ltd C_CHLORO
Coomassie brilliant blue	Sigma B0149
Ethanol	Fisher Scientific BP2818
Glacial acetic acid	Fisher Scientific 10139689
Methanol	VWR international ltd C_METHANOL_AR
PBS	Sigma P4417
Peptone water	Sigma 70179
Phenol	Sigma P1037
Potassium chloride	Sigma P9541
Sodium azide	Sigma S2002
Sodium dodecyl sulfate SDS	Sigma L3771
Sulfuric acid	Fisher Scientific 15481817
TEMED	Sigma T9281
Trifluoroacetic acid	Sigma 74564
Triton X100	Sigma X-100

Trypan blue	Sigma T6146
Virkon	Fisher Scientific 12358667
Yeast extract	Sigma 92144

4.7 Kits

Bradford assay kit	Bio-Rad 5000001
Cytotoxicity detection kitplus	Roche 11644793001
QIAamp DNA micro kit	Qiagen 56304
QIAquick PCR purification kit	Qiagen 28104

REFERENCES

Abd, H., Johansson, T., Golovliov, I., Sandström, G. and Forsman, M. (2003) 'Survival and growth of Francisella tularensis in Acanthamoeba castellanii.', *Applied and environmental microbiology*, 69(1), pp. 600–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12514047 (Accessed: 21 December 2018).

Abd, H., Saeed, A., Weintraub, A. and Sandström, G. (2009) 'Vibrio cholerae O139 requires neither capsule nor LPS O side chain to grow inside Acanthamoeba castellanii', *Journal of Medical Microbiology*, 58(1), pp. 125–131. doi: 10.1099/jmm.0.004721-0.

Abd, H., Weintraub, A. and Sandström, G. (2004) 'Interaction between Vibrio cholerae and Acanthamoeba castellanii', *Microbial Ecology in Health and Disease*. Taylor & Francis, 16(1), pp. 51–57. doi: 10.1080/08910600410029190.

Abraham, E. P. and Chain, E. (1940) 'An Enzyme from Bacteria able to Destroy Penicillin', *Nature*. Nature Publishing Group, 146(3713), pp. 837–837. doi: 10.1038/146837a0.

Abu Kwaik, Y., Gao, L. Y., Stone, B. J., Venkataraman, C. and Harb, O. S. (1998) 'Invasion of protozoa by Legionella pneumophila and its role in bacterial ecology and pathogenesis.', *Applied and environmental microbiology*. American Society for Microbiology, 64(9), pp. 3127–33. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9726849 (Accessed: 23 December 2018).

Adamska, M. (2016) 'Molecular Characterization of Acanthamoeba spp. Occurring in Water Bodies and Patients in Poland and Redefinition of Polish T16 Genotype.', *The Journal of eukaryotic microbiology*, 63(2), pp. 262–70. doi: 10.1111/jeu.12275.

Aebersold, R. and Cravatt, B. F. (2002) 'Proteomics--advances, applications and the challenges that remain.', *Trends in biotechnology*, 20(12 Suppl), pp. S1-2. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12570151 (Accessed: 29 December 2018).

Ahmed, D., Wahid, S. U. H., Sadique, T., Sultana, N., Islam, M., Halim, F., Islam, N. and Hossain, A. (2014) 'Recurrent urinary tract infection due to co-infection with extended spectrum -lactamase-producer uropathogenic Escherichia coli and enteroaggregative E. coli', *JMM Case Reports*. Society for General Microbiology, 1(3), pp. e001404–e001404. doi: 10.1099/jmmcr.0.001404.

Ahmed, H., Davies, F., Francis, N., Farewell, D., Butler, C. and Paranjothy, S. (2017) 'Long-term antibiotics for prevention of recurrent urinary tract infection in older adults: systematic review and meta-analysis of randomised trials.', *BMJ open*. British Medical Journal Publishing Group, 7(5), p. e015233. doi: 10.1136/bmjopen-2016-015233.

Akya, A., Pointon, A. and Thomas, C. (2009) 'Viability of Listeria monocytogenesâ€*ƒ*in co-culture with Acanthamoebaâ€*ƒ*spp.', *FEMS Microbiology Ecology*, 70(1), pp. 20–29. doi: 10.1111/j.1574-6941.2009.00736.x.

Al-Agamy, M. H. M., Shibl, A. M. and Tawfik, A. F. (2009) 'Prevalence and molecular characterization of extended-spectrum beta-lactamase-producing Klebsiella pneumoniae in Riyadh, Saudi Arabia.', *Annals of Saudi medicine*, 29(4), pp. 253–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19587523 (Accessed: 12 December 2018).

Al-Agamy, M. H., Shibl, A. M., Hafez, M. M., Al-Ahdal, M. N., Memish, Z. A. and Khubnani, H. (2014) 'Molecular characteristics of extended-spectrum β-lactamase-producing Escherichia coli in Riyadh: emergence of CTX-M-15-producing E. coli ST131', *Annals of Clinical Microbiology and Antimicrobials*, 13(1), p. 4. doi: 10.1186/1476-0711-13-4.

Al-Agamy, M. H., Shibl, A. M., Tawfik, A. F., Elkhizzi, N. A. and Livermore, D. M. (2012) 'Extended-spectrum and metallo-beta-lactamases among ceftazidime-resistant *Pseudomonas aeruginosa* in Riyadh, Saudi Arabia', *Journal of Chemotherapy*, 24(2), pp. 97–100. doi: 10.1179/1120009X12Z.00000000015.

Al-Badr, A. and Al-Shaikh, G. (2013) 'Recurrent Urinary Tract Infections Management in Women: A review.', *Sultan Qaboos University medical journal*, 13(3), pp. 359–67. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3749018&tool=pmcentrez&rendertype=abstract (Accessed: 7 April 2016).

Al-Herrawy, A., Bahgat, M., Mohammed, A.-E., Ashour, A. and Hikal, W. (2014) 'Acanthamoeba species in Swimming Pools of Cairo, Egypt.', *Iranian journal of parasitology*. Tehran University of Medical Sciences, 9(2), pp. 194–201. Available at: http://www.ncbi.nlm.nih.gov/pubmed/25848385 (Accessed: 13 December 2018).

Al-Kurdi, B. (2017) 'Hierarchical transcriptional profile of urothelial cells development and differentiation', *Differentiation*, 95, pp. 10–20. doi: 10.1016/j.diff.2016.10.001.

Alfieri, S. C., Correia, C. E., Motegi, S. A. and Pral, E. M. (2000) 'Proteinase activities in total extracts and in medium conditioned by Acanthamoeba polyphaga trophozoites.', *The Journal of parasitology*, 86(2), pp. 220–7. doi: 10.1645/0022-3395(2000)086[0220:PAITEA]2.0.CO;2.

Ali, A., Ali, N. S., Malik, M. B., Sayyed, Z. and Ahmad, M. Q. (2018) 'An Overview of the Pathology and Emerging Treatment Approaches for Interstitial Cystitis/Bladder Pain Syndrome', *Cureus*, 10(9), p. e3321. doi: 10.7759/cureus.3321.

Ali, A. S. M., Townes, C. L., Hall, J. and Pickard, R. S. (2009) 'Maintaining a Sterile Urinary Tract: The Role of Antimicrobial Peptides', *Journal of Urology*. American Urological Association, 182(1), pp. 21–28. doi: 10.1016/j.juro.2009.02.124.

Alizadeh, H., Pidherney, M. S., McCulley, J. P. and Niederkorn, J. Y. (1994) 'Apoptosis as a mechanism of cytolysis of tumor cells by a pathogenic free-living amoeba.', *Infection and*

immunity. American Society for Microbiology Journals, 62(4), pp. 1298–303. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8132336 (Accessed: 30 December 2018).

Alousi, S., Salloum, T., Arabaghian, H., Matar, G. M., Araj, G. F. and Tokajian, S. T. (2018) 'Genomic Characterization of MDR Escherichia coli Harboring blaOXA-48 on the IncL/M-type Plasmid Isolated from Blood Stream Infection.', *BioMed research international*. Hindawi Limited, 2018, p. 3036143. doi: 10.1155/2018/3036143.

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(6), pp. 689–694. doi: 10.1099/jmm.0.46497-0.

Alsam, S., Kim, K. S., Stins, M., Rivas, A. O., Sissons, J. and Khan, N. A. (2003) 'Acanthamoeba interactions with human brain microvascular endothelial cells', *Microbial Pathogenesis*, 35(6), pp. 235–241. doi: 10.1016/j.micpath.2003.07.001.

Alsam, S., Sissons, J., Dudley, R. and Khan, N. A. (2005) 'Mechanisms associated with Acanthamoeba castellanii (T4) phagocytosis.', *Parasitology research*, 96(6), pp. 402–9. doi: 10.1007/s00436-005-1401-z.

Alyamani, E. J., Khiyami, M. A., Booq, R. Y., Alnafjan, B. M., Altammami, M. A. and Bahwerth, F. S. (2015) 'Molecular characterization of extended-spectrum beta-lactamases (ESBLs) produced by clinical isolates of Acinetobacter baumannii in Saudi Arabia.', *Annals of clinical microbiology and antimicrobials*. BioMed Central, 14, p. 38. doi: 10.1186/s12941-015-0098-9.

Ambler, R. P. (1980) 'The structure of beta-lactamases.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 289(1036), pp. 321–31. Available at:

http://www.ncbi.nlm.nih.gov/pubmed/6109327 (Accessed: 26 October 2016).

Ambler, R. P., Coulson, A. F., Frère, J. M., Ghuysen, J. M., Joris, B., Forsman, M., Levesque, R. C., Tiraby, G. and Waley, S. G. (1991) 'A standard numbering scheme for the class A beta-lactamases.', *The Biochemical journal*, 276 (Pt 1, pp. 269–70. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1151176&tool=pmcentrez&rendertype=abstract (Accessed: 22 June 2015).

Aminov, R. I. (2010) 'A brief history of the antibiotic era: lessons learned and challenges for the future.', *Frontiers in microbiology*. Frontiers Media SA, 1, p. 134. doi: 10.3389/fmicb.2010.00134.

Ananias, M. and Yano, T. (2008) 'Serogroups and virulence genotypes of Escherichia coli isolated from patients with sepsis.', *Brazilian journal of medical and biological research = Revista brasileira de pesquisas medicas e biologicas*, 41(10), pp. 877–83. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19030710 (Accessed: 31 December 2018).

Anderson, G., DODSON, K., HOOTON, T. and HULTGREN, S. (2004) 'Intracellular bacterial communities of uropathogenic in urinary tract pathogenesis', *Trends in Microbiology*, 12(9), pp. 424–430. doi: 10.1016/j.tim.2004.07.005.

Arnalich-Montiel, F., Lumbreras-Fernández, B., Martín-Navarro, C. M., Valladares, B., Lopez-Velez, R., Morcillo-Laiz, R. and Lorenzo-Morales, J. (2014) 'Influence of Acanthamoeba genotype on clinical course and outcomes for patients with Acanthamoeba keratitis in Spain.', *Journal of clinical microbiology*, 52(4), pp. 1213–6. doi: 10.1128/JCM.00031-14.

Arnaud, I., Maugat, S., Jarlier, V., Astagneau, P. and National Early Warning, Investigation and Surveillance of Healthcare-Associated Infections Network (RAISIN)/multidrug resistance study group (2015) 'Ongoing increasing temporal and geographical trends of the incidence

of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* infections in France, 2009 to 2013', *Eurosurveillance*, 20(36), p. 30014. doi: 10.2807/1560-7917.ES.2015.20.36.30014.

Arritt, F. M., Eifert, J. D., Pierson, M. D. and Sumner, S. S. (2002) 'Efficacy of Antimicrobials Against Campylobacter jejuni on Chicken Breast Skin', *The Journal of Applied Poultry Research*. Oxford University Press, 11(4), pp. 358–366. doi: 10.1093/japr/11.4.358.

Astorga, B., Lorenzo-Morales, J., Martín-Navarro, C. M., Alarcón, V., Moreno, J., González, A. C., Navarrete, E., Piñero, J. E. and Valladares, B. (2011) 'Acanthamoeba Belonging to T3, T4, and T11: Genotypes Isolated from Air-Conditioning Units in Santiago, Chile', *Journal of Eukaryotic Microbiology*, 58(6), pp. 542–544. doi: 10.1111/j.1550-7408.2011.00584.x.

Auer, S., Wojna, A. and Hell, M. (2010) 'Oral treatment options for ambulatory patients with urinary tract infections caused by extended-spectrum-beta-lactamase-producing Escherichia coli.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology Journals, 54(9), pp. 4006–8. doi: 10.1128/AAC.01760-09.

Bäckhed, F., Söderhäll, M., Ekman, P., Normark, S. and Richter-Dahlfors, A. (2001) 'Induction of innate immune responses by Escherichia coli and purified lipopolysaccharide correlate with organ- and cell-specific expression of Toll-like receptors within the human urinary tract.', *Cellular microbiology*, 3(3), pp. 153–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11260138 (Accessed: 26 May 2015).

Bahrani-Mougeot, F. K., Buckles, E. L., Lockatell, C. V, Hebel, J. R., Johnson, D. E., Tang, C. M. and Donnenberg, M. S. (2002) 'Type 1 fimbriae and extracellular polysaccharides are preeminent uropathogenic Escherichia coli virulence determinants in the murine urinary tract.', *Molecular microbiology*, 45(4), pp. 1079–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12180926 (Accessed: 24 December 2018).

Baig, A. M., Zohaib, R., Nuzair, W., Saiqa, K. and Mehdia, N. (2018) 'Evidence of human-like Ca $^{2+}$ Channels and Effects of Ca $^{2+}$ Channel blockers in *Acanthamoeba castellanii*', *Chemical Biology & Drug Design*. doi: 10.1111/cbdd.13421.

Baird, B. E., Lucia, L. M., Acuff, G. R., Harris, K. B. and Savell, J. W. (2006) 'Beef hide antimicrobial interventions as a means of reducing bacterial contamination', *Meat Science*, 73(2), pp. 245–248. doi: 10.1016/j.meatsci.2005.11.023.

Bajaj, P., Singh, N. S. and Virdi, J. S. (2016) 'Escherichia coli β-Lactamases: What Really Matters.', *Frontiers in microbiology*. Frontiers Media SA, 7, p. 417. doi: 10.3389/fmicb.2016.00417.

Bang, C. S., Kinnunen, A., Karlsson, M., Önnberg, A., Söderquist, B. and Persson, K. (2014) 'The antibacterial effect of nitric oxide against ESBL-producing uropathogenic E. coli is improved by combination with miconazole and polymyxin B nonapeptide.', *BMC microbiology*. BioMed Central, 14(1), p. 65. doi: 10.1186/1471-2180-14-65.

Baraniak, A., Izdebski, R., Fiett, J., Sadowy, E., Adler, A., Kazma, M., Salomon, J., Lawrence, C., Rossini, A., Salvia, A., Vidal Samso, J., Fierro, J., Paul, M., Lerman, Y., Malhotra-Kumar, S., Lammens, C., Goossens, H., Hryniewicz, W., Brun-Buisson, C., Carmeli, Y., Gniadkowski, M. and MOSAR WP2 and WP5 Study Groups (2013) 'Comparative population analysis of Klebsiella pneumoniae strains with extended-spectrum β-lactamases colonizing patients in rehabilitation centers in four countries.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 57(4), pp. 1992–7. doi: 10.1128/AAC.02571-12.

Barber, A. E., Norton, J. P., Spivak, A. M. and Mulvey, M. A. (2013) 'Urinary Tract Infections: Current and Emerging Management Strategies', *Clinical Infectious Diseases*, 57(5), pp. 719–724. doi: 10.1093/cid/cit284.

Barker, J., Brown, M. R., Collier, P. J., Farrell, I. and Gilbert, P. (1992) 'Relationship between Legionella pneumophila and Acanthamoeba polyphaga: physiological status and susceptibility to chemical inactivation.', *Applied and environmental microbiology*, 58(8), pp. 2420–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1514790 (Accessed: 1 January 2019).

Barker, J., Humphrey, T. J. and Brown, M. W. . (1999) 'Survival of *Escherichia coli* 0157 in a soil protozoan: implications for disease', *FEMS Microbiology Letters*, 173(2), pp. 291–295. doi: 10.1111/j.1574-6968.1999.tb13516.x.

Basilico, N., Migotto, M., Ilboudo, D. P., Taramelli, D., Stradi, R. and Pini, E. (2015) 'Modified quaternary ammonium salts as potential antimalarial agents', *Bioorganic & Medicinal Chemistry*. Pergamon, 23(15), pp. 4681–4687. doi: 10.1016/J.BMC.2015.05.055.

Beahm, N. P., Nicolle, L. E., Bursey, A., Smyth, D. J. and Tsuyuki, R. T. (2017) 'The assessment and management of urinary tract infections in adults: Guidelines for pharmacists.', *Canadian pharmacists journal : CPJ = Revue des pharmaciens du Canada : RPC*. SAGE Publications, 150(5), pp. 298–305. doi: 10.1177/1715163517723036.

Beerepoot, M. and Geerlings, S. (2016) 'Non-Antibiotic Prophylaxis for Urinary Tract Infections', *Pathogens*, 5(2), p. 36. doi: 10.3390/pathogens5020036.

Beers, K., Rheingans, J., Chinault, K., Cook, P., Smith, B. and Waldroup, A. (2006) 'Microbial efficacy of commercial application of Cecure® CPC antimicrobial to ingest contaminated pre-chill broiler carcasses', *International Journal of Poultry Science*. Asian Network for Scientific Information, (5), pp. 698–703. Available at: http://agris.fao.org/agris-search/search.do?recordID=DJ2012051716 (Accessed: 1 January 2019).

Behera, H. S., Panda, A., Satpathy, G., Bandivadekar, P., Vanathi, M., Agarwal, T., Nayak, N.

and Tandon, R. (2016) ‘Genotyping of Acanthamoeba spp. and characterization of the prevalent T4 type along with T10 and unassigned genotypes from amoebic keratitis (AK) patients in India.’, *Journal of medical microbiology*. doi: 10.1099/jmm.0.000234.

Behzadi, P., Behzadi, E., Yazdanbod, H., Aghapour, R., Akbari Cheshmeh, M. and Salehian Omran, D. (2010) ‘A survey on urinary tract infections associated with the three most common uropathogenic bacteria.’, *Maedica*. Amaltea Medical, Editura Magister, 5(2), pp. 111–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21977133 (Accessed: 12 December 2018).

Bellamy, W. D. and Klimek, J. W. (1948) ‘Some Properties of Penicillin-resistant Staphylococci.’, *Journal of bacteriology*. American Society for Microbiology (ASM), 55(2), pp. 153–60. Available at: http://www.ncbi.nlm.nih.gov/pubmed/16561443 (Accessed: 11 December 2018).

Bennett, T. L., Kraft, S. M., Reaves, B. J., Mima, J., O’Brien, K. M. and Starai, V. J. (2013) ‘LegC3, an Effector Protein from Legionella pneumophila, Inhibits Homotypic Yeast Vacuole Fusion In Vivo and In Vitro’, *PLoS ONE*. Edited by D. Davis, 8(2), p. e56798. doi: 10.1371/journal.pone.0056798.

Bereswill, S., Vey, T. and Kist, M. (1999) ‘Susceptibility in vitro of Helicobacter pylori to cetylpyridinium chloride’, *FEMS Immunology & Medical Microbiology*. The Oxford University Press, 24(2), pp. 189–192. doi: 10.1111/j.1574-695X.1999.tb01281.x.

Berry, R. E., Klumpp, D. J. and Schaeffer, A. J. (2009) ‘Urothelial Cultures Support Intracellular Bacterial Community Formation by Uropathogenic Escherichia coli’, *Infection and Immunity*, 77(7), pp. 2762–2772. doi: 10.1128/IAI.00323-09.

Beutin, L. (1991) ‘The different hemolysins of Escherichia coli.’, *Medical microbiology and*

immunology, 180(4), pp. 167–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1784268 (Accessed: 1 January 2019).

Beveridge, T. J. (1999) 'Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles', *J. Bacteriol.*, 181(16), pp. 4725–4733. Available at: http://jb.asm.org/content/181/16/4725.full (Accessed: 25 May 2015).

Beytur, A., Yakupogullari, Y., Oguz, F., Otlu, B. and Kaysadu, H. (2015) 'Oral amoxicillin-clavulanic Acid treatment in urinary tract infections caused by extended-spectrum Beta-lactamase-producing organisms.', *Jundishapur journal of microbiology*, 8(1), p. e13792. doi: 10.5812/jjm.13792.

Bien, J., Sokolova, O. and Bozko, P. (2012) 'Role of uropathogenic escherichia coli virulence factors in development of urinary tract infection and kidney damage', *International Journal of Nephrology*. doi: 10.1155/2012/681473.

Binns, M. M., Mayden, J. and Levine, R. P. (1982) 'Further characterization of complement resistance conferred on Escherichia coli by the plasmid genes traT of R100 and iss of ColV,I-K94.', *Infection and immunity*. American Society for Microbiology (ASM), 35(2), pp. 654–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7035371 (Accessed: 24 December 2018).

Bloch, K. C. and Schuster, F. L. (2005) 'Inability to make a premortem diagnosis of Acanthamoeba species infection in a patient with fatal granulomatous amebic encephalitis.', *Journal of clinical microbiology*. American Society for Microbiology (ASM), 43(6), pp. 3003–6. doi: 10.1128/JCM.43.6.3003-3006.2005.

Blum, G., Ott, M., Cross, A. and Hacker, J. (1991) 'Virulence determinants of Escherichia coli O6 extraintestinal isolates analysed by Southern hybridizations and DNA long range

mapping techniques.', *Microbial pathogenesis*, 10(2), pp. 127–36. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1679907 (Accessed: 23 December 2018).

Blumberg, J. B., Camesano, T. A., Cassidy, A., Kris-Etherton, P., Howell, A., Manach, C., Ostertag, L. M., Sies, H., Skulas-Ray, A. and Vita, J. A. (2013) 'Cranberries and Their Bioactive Constituents in Human Health', *Advances in Nutrition*. Oxford University Press, 4(6), pp. 618–632. doi: 10.3945/an.113.004473.

Bonnet, R. (2004) 'Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes.', *Antimicrobial agents and chemotherapy*, 48(1), pp. 1–14. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=310187&tool=pmcentrez&rendertype=abstract (Accessed: 11 March 2015).

Bottone, E. J., Pere, A. A., Gordon, R. E. and Qureshi, M. N. (1994) 'Differential binding capacity and internalisation of bacterial substrates as factors in growth rate of Acanthamoeba spp.', *Journal of Medical Microbiology*, 40(2), pp. 148–154. doi: 10.1099/00222615-40-2-148.

Bou-Antoun, S., Davies, J., Guy, R., Johnson, A. P., Sheridan, E. A. and Hope, R. J. (2016) 'Descriptive epidemiology of Escherichia coli bacteraemia in England, April 2012 to March 2014.', *Euro surveillance : bulletin Europeen sur les maladies transmissibles = European communicable disease bulletin*. European Centre for Disease Prevention and Control, 21(35). doi: 10.2807/1560-7917.ES.2016.21.35.30329.

Le Bouguénec, C., Lalioui, L., du Merle, L., Jouve, M., Courcoux, P., Bouzari, S., Selvarangan, R., Nowicki, B. J., Germani, Y., Andremont, A., Gounon, P. and Garcia, M. I. (2001) 'Characterization of AfaE adhesins produced by extraintestinal and intestinal human Escherichia coli isolates: PCR assays for detection of Afa adhesins that do or do not recognize Dr blood group antigens.', *Journal of clinical microbiology*. American Society

for Microbiology (ASM), 39(5), pp. 1738–45. doi: 10.1128/JCM.39.5.1738-1745.2001.

Le Bouguénec, C. and Servin, A. L. (2006) 'Diffusely adherent Escherichia coli strains expressing Afa/Dr adhesins (Afa/Dr DAEC): Hitherto unrecognized pathogens', *FEMS Microbiology Letters*, 256(2), pp. 185–194. doi: 10.1111/j.1574-6968.2006.00144.x.

Bower, J. M., Eto, D. S. and Mulvey, M. A. (2005) 'Covert Operations of Uropathogenic Escherichia coli within the Urinary Tract', *Traffic*, 6(1), pp. 18–31. doi: 10.1111/j.1600-0854.2004.00251.x.

Bowers, B. and Korn, E. D. (1968) 'The fine structure of Acanthamoeba castellanii. I. The trophozoite.', *The Journal of cell biology*, 39(1), pp. 95–111. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2107510&tool=pmcentrez&rendertype=abstract (Accessed: 28 May 2015).

Bowers, B. and Olszewski, T. E. (1972) 'Pinocytosis in Acanthamoeba castellanii. Kinetics and morphology.', *The Journal of cell biology*, 53(3), pp. 681–94. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=2108769&tool=pmcentrez&rendertype=abstract (Accessed: 28 May 2015).

Bradbury, R. S., French, L. P. and Blizzard, L. (2014) 'Prevalence of Acanthamoeba spp. in Tasmanian intensive care clinical specimens', *Journal of Hospital Infection*, 86(3), pp. 178–181. doi: 10.1016/j.jhin.2013.12.009.

Bradford, P. A. (2001) 'Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat.', *Clinical microbiology reviews*, 14(4), p. 933–51, table of contents. doi: 10.1128/CMR.14.4.933-951.2001.

Brasier, A. R., Garcia, J., Wiktorowicz, J. E., Spratt, H. M., Comach, G., Ju, H., Recinos, A., Soman, K., Forshey, B. M., Halsey, E. S., Blair, P. J., Rocha, C., Bazan, I., Victor, S. S.,

Wu, Z., Stafford, S., Watts, D., Morrison, A. C., Scott, T. W., Kochel, T. J. and Venezuelan Dengue Fever Working Group (2012) 'Discovery Proteomics and Nonparametric Modeling Pipeline in the Development of a Candidate Biomarker Panel for Dengue Hemorrhagic Fever', *Clinical and Translational Science*, 5(1), pp. 8–20. doi: 10.1111/j.1752-8062.2011.00377.x.

Brioschi, M., Lento, S., Tremoli, E. and Banfi, C. (2013) 'Proteomic analysis of endothelial cell secretome: A means of studying the pleiotropic effects of Hmg-CoA reductase inhibitors', *Journal of Proteomics*, 78, pp. 346–361. doi: 10.1016/j.jprot.2012.10.003.

Brolund, A., Edquist, P. J., Mäkitalo, B., Olsson-Liljequist, B., Söderblom, T., Tegmark Wisell, K. and Giske, C. G. (2014) 'Epidemiology of extended-spectrum β-lactamase-producing Escherichia coli in Sweden 2007–2011', *Clinical Microbiology and Infection*, 20(6), pp. O344–O352. doi: 10.1111/1469-0691.12413.

Brooks, T. and Keevil, C. W. (1997) 'A simple artificial urine for the growth of urinary pathogens', *Letters in Applied Microbiology*. Blackwell Science Ltd, 24(3), pp. 203–206. doi: 10.1046/j.1472-765X.1997.00378.x.

Brown, A. G., Butterworth, D., Cole, M., Hanscomb, G., Hood, J. D., Reading, C. and Rolinson, G. N. (1976) 'Naturally-occurring beta-lactamase inhibitors with antibacterial activity.', *The Journal of antibiotics*, 29(6), pp. 668–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/950324 (Accessed: 11 December 2018).

Brown, D., Alvarado, C. and A., J. (2018) *Sepsis (Septic), Peritonitis, StatPearls*. StatPearls Publishing. Available at: http://www.ncbi.nlm.nih.gov/pubmed/30252385 (Accessed: 10 December 2018).

Bui, X., Qvortrup, K., Wolff, A., Bang, D. and Creuzenet, C. (2012) 'Effect of environmental

stress factors on the uptake and survival of Campylobacter jejuni in Acanthamoeba castellanii', *BMC Microbiology*. BioMed Central, 12(1), p. 232. doi: 10.1186/1471-2180-12-232.

Burke, L., Humphreys, H. and Fitzgerald-Hughes, D. (2012) 'The revolving door between hospital and community: extended-spectrum beta-lactamase-producing Escherichia coli in Dublin', *Journal of Hospital Infection*, 81(3), pp. 192–198. doi: 10.1016/j.jhin.2012.04.021.

Burman, L. G., Park, J. T., Lindström, E. B. and Boman, H. G. (1973) 'Resistance of Escherichia coli to penicillins: identification of the structural gene for the chromosomal penicillinase.', *Journal of bacteriology*, 116(1), pp. 123–30. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4200837 (Accessed: 12 December 2018).

Bush, Jacoby and Medeiros (1995) 'A functional classification scheme for beta-lactamases and its correlation with molecular structure.', *Antimicrobial agents and chemotherapy*, 39(6), pp. 1211–33. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=162717&tool=pmcentrez&rendertype=abstract (Accessed: 1 April 2015).

Bush, K. and Jacoby, G. A. (2010) 'Updated Functional Classification of -Lactamases', *Antimicrobial Agents and Chemotherapy*, 54(3), pp. 969–976. doi: 10.1128/AAC.01009-09.

Bush, K., Jacoby, G. A. and Medeiros, A. A. (1995) 'A functional classification scheme for beta-lactamases and its correlation with molecular structure.', *Antimicrobial agents and chemotherapy*, 39(6), pp. 1211–1233. doi: 10.1128/AAC.39.6.1211.

Bush, K., Palzkill, T. and Jacoby, G. (2015) *ß-Lactamase Classification and Amino Acid Sequences for TEM, SHV and OXA Extended-Spectrum and Inhibitor Resistant Enzymes*. Available at: https://www.lahey.org/Studies/ (Accessed: 12 December 2018).

Cakirlar, F. K., Ciftci, I. H. and Gonullu, N. (2015) 'OXA-type Carbapenemases and Susceptibility of Colistin and Tigecycline Among Carbapenem-Resistant Acinetobacter Baumannii Isolates from Patients with Bacteremia in Turkey.', *Clinical laboratory*, 61(7), pp. 741–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26299073 (Accessed: 12 December 2018).

Calzada, E., Onguka, O. and Claypool, S. M. (2016) 'Phosphatidylethanolamine Metabolism in Health and Disease.', *International review of cell and molecular biology*. NIH Public Access, 321, pp. 29–88. doi: 10.1016/bs.ircmb.2015.10.001.

Candan, E. D., Aksöz, N., Candan, E. D. and Aksöz, N. (2017) 'Escherichia Coli: Characteristics of Carbapenem Resistance and Virulence Factors', *Brazilian Archives of Biology and Technology*. Tecpar, 60(0). doi: 10.1590/1678-4324-2017160416.

Cantón, R., Akóva, M., Carmeli, Y., Giske, C. G., Glupczynski, Y., Gniadkowski, M., Livermore, D. M., Miriagou, V., Naas, T., Rossolini, G. M., Samuelsen, Ø., Seifert, H., Woodford, N., Nordmann, P. and European Network on Carbapenemases (2012) 'Rapid evolution and spread of carbapenemases among Enterobacteriaceae in Europe', *Clinical Microbiology and Infection*, 18(5), pp. 413–431. doi: 10.1111/j.1469-0691.2012.03821.x.

Caprioli, A., Falbo, V., Roda, L. G., Ruggeri, F. M. and Zona, C. (1983) 'Partial purification and characterization of an escherichia coli toxic factor that induces morphological cell alterations.', *Infection and immunity*. American Society for Microbiology (ASM), 39(3), pp. 1300–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6341235 (Accessed: 1 January 2019).

Cardas, M. (2014) *Aspects of the clinical and environmental implications of interactions between Staphylococci and Acanthamoeba*. university of essex.

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(4), pp. 513–8. doi: 10.1016/j.exppara.2012.08.014.

Care, C., Points, K., Problem, C. and Dilemma, C. (2005) ‘Adult women with uncomplicated UTI’, *University of Michigan,Health System*, pp. 1–9.

Carrer, A., Poirel, L., Yilmaz, M., Akan, O. A., Feriha, C., Cuzon, G., Matar, G., Honderlick, P. and Nordmann, P. (2010) ‘Spread of OXA-48-Encoding Plasmid in Turkey and Beyond’, *Antimicrobial Agents and Chemotherapy*, 54(3), pp. 1369–1373. doi: 10.1128/AAC.01312-09.

Castanheira, M., Deshpande, L. M., Mathai, D., Bell, J. M., Jones, R. N. and Mendes, R. E. (2011) ‘Early dissemination of NDM-1- and OXA-181-producing Enterobacteriaceae in Indian hospitals: Report from the SENTRY Antimicrobial Surveillance Program, 2006-2007’, *Antimicrobial Agents and Chemotherapy*, 55(3), pp. 1274–1278. doi: 10.1128/AAC.01497-10.

Castro-Artavia, E., Retana-Moreira, L., Lorenzo-Morales, J. and Abrahams-Sandí, E. (2017) ‘Potentially pathogenic Acanthamoeba genotype T4 isolated from dental units and emergency combination showers’, *Memórias do Instituto Oswaldo Cruz*, 112(12), pp. 817–821. doi: 10.1590/0074-02760170147.

Cave, D., Alfonso, R., Comlavi, K. A., Orazi, C., Monno, R. and Berrilli, F. (2014) ‘Genotypic heterogeneity based on 18S-rRNA gene sequences among Acanthamoeba isolates from clinical samples in Italy’, *Experimental Parasitology*, 145, pp. S46–S49. doi: 10.1016/j.exppara.2014.05.009.

Chan, F. K.-M., Moriwaki, K. and De Rosa, M. J. (2013) ‘Detection of Necrosis by Release of

Lactate Dehydrogenase Activity', in *Methods in molecular biology (Clifton, N.J.)*, pp. 65–70. doi: 10.1007/978-1-62703-290-2_7.

Chandrdas, D., Jayakumar, H. L., Chandra, M., Katodia, L. and Sreedevi, A. (2014) 'Evaluation of antimicrobial efficacy of garlic, tea tree oil, cetylpyridinium chloride, chlorhexidine, and ultraviolet sanitizing device in the decontamination of toothbrush.', *Indian journal of dentistry*, 5(4), pp. 183–9. doi: 10.4103/0975-962X.144718.

Chapman, E. J., Kelly, G. and Knowles, M. A. (2008) 'Genes involved in differentiation, stem cell renewal, and tumorigenesis are modulated in telomerase-immortalized human urothelial cells.', *Molecular cancer research : MCR*, 6(7), pp. 1154–68. doi: 10.1158/1541-7786.MCR-07-2168.

Chevallet, M., Luche, S. and Rabilloud, T. (2006) 'Silver staining of proteins in polyacrylamide gels.', *Nature protocols*, 1(4), pp. 1852–8. doi: 10.1038/nprot.2006.288.

Chroma, M. and Kolar, M. (2010) 'Genetic methods for detection of antibiotic resistance: focus on extended-spectrum β-lactamases.', *Biomedical papers of the Medical Faculty of the University Palacký, Olomouc, Czechoslovakia*, 154(4), pp. 289–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21293539.

Chusattayanond, A. D., Boonsilp, S., Kasisit, J., Boonmee, A. and Warit, S. (2010) 'Thai Acanthamoeba isolate (T4) induced apoptotic death in neuroblastoma cells via the Bax-mediated pathway', *Parasitology International*, 59(4), pp. 512–516. doi: 10.1016/j.parint.2010.06.007.

Cirillo, J. D., Falkow, S. and Tompkins, L. S. (1994) 'Growth of Legionella pneumophila in Acanthamoeba castellanii enhances invasion.', *Infection and immunity*, 62(8), pp. 3254–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8039895 (Accessed: 1 January 2019).

Cirillo, J. D., Falkow, S., Tompkins, L. S. and Bermudez, L. E. (1997) 'Interaction of Mycobacterium avium with environmental amoebae enhances virulence.', *Infection and immunity*, 65(9), pp. 3759–67. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9284149 (Accessed: 13 December 2018).

Conover, M. S., Hadjifrangiskou, M., Palermo, J. J., Hibbing, M. E., Dodson, K. W. and Hultgren, S. J. (2016) 'Metabolic Requirements of Escherichia coli in Intracellular Bacterial Communities during Urinary Tract Infection Pathogenesis.', *mBio*. American Society for Microbiology, 7(2), pp. e00104-16. doi: 10.1128/mBio.00104-16.

Cooke, N. M., Smith, S. G., Kelleher, M. and Rogers, T. R. (2010) 'Major differences exist in frequencies of virulence factors and multidrug resistance between community and nosocomial Escherichia coli bloodstream isolates.', *Journal of clinical microbiology*. American Society for Microbiology (ASM), 48(4), pp. 1099–104. doi: 10.1128/JCM.02017-09.

Corvec, S., Caroff, N., Espaze, E., Marraillac, J. and Reynaud, A. (2002) '-11 Mutation in the ampC promoter increasing resistance to beta-lactams in a clinical Escherichia coli strain.', *Antimicrobial agents and chemotherapy*, 46(10), pp. 3265–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=128767&tool=pmcentrez&rendertype=abstract (Accessed: 17 August 2015).

Coven, S. L., Song, E., Steward, S., Pierson, C. R., Cope, J. R., Ali, I. K., Ardura, M. I., Hall, M. W., Chung, M. G. and Bajwa, R. P. S. (2017) 'Acanthamoeba granulomatous amoebic encephalitis after pediatric hematopoietic stem cell transplant', *Pediatric Transplantation*, 21(8), p. e13060. doi: 10.1111/petr.13060.

Croxall, G., Weston, V., Joseph, S., Manning, G., Cheetham, P. and McNally, A. (2011) 'Increased human pathogenic potential of Escherichia coli from polymicrobial urinary tract

infections in comparison to isolates from monomicrobial culture samples.', *Journal of medical microbiology*, 60(Pt 1), pp. 102–9. doi: 10.1099/jmm.0.020602-0.

Cruz, J., Figueiredo, F., Matos, A. P., Duarte, S., Guerra, A. and Ramalho, M. (2019) 'Infectious and Inflammatory Diseases of the Urinary Tract', *Magnetic Resonance Imaging Clinics of North America*, 27(1), pp. 59–75. doi: 10.1016/j.mric.2018.09.001.

Cutter, C. N., Dorsa, W. J., Handie, A., Rodriguez-Morales, S., Zhou, X., Breen, P. J. and Compadre, C. M. (2000) 'Antimicrobial activity of cetylpyridinium chloride washes against pathogenic bacteria on beef surfaces.', *Journal of food protection*, 63(5), pp. 593–600. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10826715 (Accessed: 27 October 2016).

Cuzon, G., Ouanich, J., Gondret, R., Naas, T. and Nordmann, P. (2011) 'Outbreak of OXA-48-positive carbapenem-resistant Klebsiella pneumoniae isolates in France', *Antimicrobial Agents and Chemotherapy*, 55(5), pp. 2420–2423. doi: 10.1128/AAC.01452-10.

Darnton, N. C., Turner, L., Rojevsky, S. and Berg, H. C. (2007) 'On torque and tumbling in swimming Escherichia coli.', *Journal of bacteriology*, 189(5), pp. 1756–64. doi: 10.1128/JB.01501-06.

Dason, S., Dason, J. T. and Kapoor, A. (2011) 'Guidelines for the diagnosis and management of recurrent urinary tract infection in women.', *Canadian Urological Association journal = Journal de l'Association des urologues du Canada*. Canadian Medical Association, 5(5), pp. 316–22. doi: 10.5489/cuaj.11214.

Datta, N. and Kontomichalou, P. (1965) 'Penicillinase synthesis controlled by infectious R factors in Enterobacteriaceae.', *Nature*, 208(5007), pp. 239–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/5326330 (Accessed: 23 June 2015).

Davies, J. and Davies, D. (2010) 'Origins and evolution of antibiotic resistance.', *Microbiology*

and molecular biology reviews : MMBR. American Society for Microbiology (ASM), 74(3), pp. 417–33. doi: 10.1128/MMBR.00016-10.

Davis, N. and Hugh, F. (1999) 'The Pathogenesis of Urinary Tract Infections', *Pediatric annals*, 28(10), pp. 639–642.

Declerck, P., Vanysacker, L., Hulsmans, A., Lambert, N., Liers, S. and Ollevier, F. (2010) 'Evaluation of power ultrasound for disinfection of both Legionella pneumophila and its environmental host Acanthamoeba castellanii', *Water Research*, 44(3), pp. 703–710. doi: 10.1016/j.watres.2009.09.062.

Delcour, A. H. (2009) 'Outer membrane permeability and antibiotic resistance.', *Biochimica et biophysica acta*, 1794(5), pp. 808–16. doi: 10.1016/j.bbapap.2008.11.005.

Delzell, J. J. and Lefevre, M. (2000) 'Urinary Tract Infections During Pregnancy', *American Family Physician*, 61(3), pp. 713–720.

Dendana, F., Sellami, H., Jarraya, F., Sellami, A., Makni, F., Cheikhrouhou, F., Hachicha, J. and Ayadi, A. (2008) 'Free-living amoebae (FLA): detection, morphological and molecular identification of Acanthamoeba genus in the hydraulic system of an haemodialysis unit in Tunisia.', *Parasite (Paris, France)*, 15(2), pp. 137–42. doi: 10.1051/parasite/2008152137.

Dereeper, A., Guignon, V., Blanc, G., Audic, S., Buffet, S., Chevenet, F., Dufayard, J.-F., Guindon, S., Lefort, V., Lescot, M., Claverie, J.-M. and Gascuel, O. (2008) 'Phylogeny.fr: robust phylogenetic analysis for the non-specialist', *Nucleic Acids Research*, 36(Web Server), pp. W465–W469. doi: 10.1093/nar/gkn180.

Dewar, S., Reed, L. C. and Koerner, R. J. (2014) 'Emerging clinical role of pivmecillinam in the treatment of urinary tract infection in the context of multidrug-resistant bacteria', *Journal of Antimicrobial Chemotherapy*, 69(2), pp. 303–308. doi: 10.1093/jac/dkt368.

Dhillon, R. H. P. and Clark, J. (2012) 'ESBLs: A clear and present danger?', *Critical Care Research and Practice*. doi: 10.1155/2012/625170.

Dobrindt, U., Blum-Oehler, G., Nagy, G., Schneider, G., Johann, A., Gottschalk, G. and Hacker, J. (2002) 'Genetic structure and distribution of four pathogenicity islands (PAI I(536) to PAI IV(536)) of uropathogenic Escherichia coli strain 536.', *Infection and immunity*, 70(11), pp. 6365–72. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12379716 (Accessed: 31 December 2018).

Doğru, A., Karatoka, B., Ergen, P., Şen Aydın, Ö. and Tigen, E. T. (2013) 'The resistance rates of urinary tract infections: Our data from year 2010.', *Turkish journal of urology*. Turkish Association of Urology, 39(4), pp. 237–43. doi: 10.5152/tud.2013.060.

Drawz, S. M. and Bonomo, R. A. (2010) 'Three Decades of -Lactamase Inhibitors', *Clinical Microbiology Reviews*, 23(1), pp. 160–201. doi: 10.1128/CMR.00037-09.

Drawz, S. M., Papp-Wallace, K. M. and Bonomo, R. A. (2014) 'New β-Lactamase Inhibitors: a Therapeutic Renaissance in an MDR World', *Antimicrobial Agents and Chemotherapy*, 58(4), pp. 1835–1846. doi: 10.1128/AAC.00826-13.

Drozanski, W. (1952) 'Fatal bacterial infection in soil amoebae.', *Acta microbiologica Polonica*, 5(3–4), pp. 315–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/13410554 (Accessed: 30 December 2018).

Dudley, R., Alsam, S. and Khan, N. A. (2008) 'The role of proteases in the differentiation of Acanthamoeba castellanii', *FEMS Microbiology Letters*, 286(1), pp. 9–15. doi: 10.1111/j.1574-6968.2008.01249.x.

van Duin, D. and Doi, Y. (2017) 'The global epidemiology of carbapenemase-producing Enterobacteriaceae', *Virulence*, 8(4), pp. 460–469. doi: 10.1080/21505594.2016.1222343.

Eberspächer, B., Hugo, F. and Bhakdi, S. (1989) 'Quantitative study of the binding and hemolytic efficiency of Escherichia coli hemolysin.', *Infection and immunity*, 57(3), pp. 983–8. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=313209&tool=pmcentrez&rendertype=abstract (Accessed: 27 May 2015).

Ejrnæs, K. (2011) 'Bacterial characteristics of importance for recurrent urinary tract infections caused by Escherichia coli.', *Danish medical bulletin*, 58(4), p. B4187. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21466767 (Accessed: 21 December 2018).

El-Mahdy, T. S., Al-Agamy, M. H., Al-Qahtani, A. A. and Shibl, A. M. (2017) 'Detection of *bla* $_{\text{OXA-23-like}}$ and *bla* $_{\text{NDM-1}}$ in *Acinetobacter baumannii* from the Eastern Region, Saudi Arabia', *Microbial Drug Resistance*, 23(1), pp. 115–121. doi: 10.1089/mdr.2015.0304.

Evans, B. A. and Amyes, S. G. B. (2014) 'OXA β-lactamases.', *Clinical microbiology reviews*, 27(2), pp. 241–63. doi: 10.1128/CMR.00117-13.

Fair, R. J. and Tor, Y. (2014) 'Antibiotics and bacterial resistance in the 21st century.', *Perspectives in medicinal chemistry*. SAGE Publications, 6, pp. 25–64. doi: 10.4137/PMC.S14459.

Falagas, M. E. and Karageorgopoulos, D. E. (2009) 'Extended-spectrum β-lactamase-producing organisms', *Journal of Hospital Infection*, 73(4), pp. 345–354. doi: 10.1016/j.jhin.2009.02.021.

Farshadzadeh, Z., Khosravi, A. D., Alavi, S. M., Parhizgari, N. and Hoveizavi, H. (2014) 'Spread of extended-spectrum β-lactamase genes of bla OXA-10, bla PER-1 and bla CTX-M in Pseudomonas aeruginosa strains isolated from burn patients', *Burns*, 40(8), pp. 1575–1580. doi: 10.1016/j.burns.2014.02.008.

Feres, M., Figueiredo, L. C., Faveri, M., Stewart, B. and de Vizio, W. (2010) 'The effectiveness of a preprocedural mouthrinse containing cetylpyridinium chloride in reducing bacteria in the dental office.', *Journal of the American Dental Association (1939)*, 141(4), pp. 415–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20354090 (Accessed: 1 January 2019).

Fischer, H., Ellström, P., Ekström, K., Gustafsson, L., Gustafsson, M. and Svanborg, C. (2007) 'Ceramide as a TLR4 agonist; a putative signalling intermediate between sphingolipid receptors for microbial ligands and TLR4.', *Cellular microbiology*, 9(5), pp. 1239–51. doi: 10.1111/j.1462-5822.2006.00867.x.

Fisher, J. F., Meroueh, S. O. and Mobashery, S. (2005) 'Bacterial resistance to β-lactam antibiotics: Compelling opportunism, compelling opportunity', *Chemical Reviews*, 105(2), pp. 395–424. doi: 10.1021/cr030102i.

Flores-Mireles, A. L., Walker, J. N., Caparon, M. and Hultgren, S. J. (2015) 'Urinary tract infections: epidemiology, mechanisms of infection and treatment options', *Nature Reviews Microbiology*. Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 13(5), pp. 269–284. doi: 10.1038/nrmicro3432.

Fotadar, U., Zaveloff, P. and Terracio, L. (2005) 'Growth ofEscherichia coli at elevated temperatures', *Journal of Basic Microbiology*, 45(5), pp. 403–404. doi: 10.1002/jobm.200410542.

Foxman, B. (2010) 'The epidemiology of urinary tract infection.', *Nature reviews. Urology*, 7(12), pp. 653–60. doi: 10.1038/nrurol.2010.190.

Foxman, B. and Buxton, M. (2013) 'Alternative Approaches to Conventional Treatment of Acute Uncomplicated Urinary Tract Infection in Women', *Current Infectious Disease Reports*, 15(2), pp. 124–129. doi: 10.1007/s11908-013-0317-5.

Frère, J.-M., Sauvage, E. and Kerff, F. (2016) ‘From "An Enzyme Able to Destroy Penicillin" to Carbapenemases: 70 Years of Beta-lactamase Misbehaviour.’, *Current drug targets*, 17(9), pp. 974–82. Available at: http://www.ncbi.nlm.nih.gov/pubmed/26424390 (Accessed: 11 December 2018).

Fukumoto, T., Matsuo, J., Okubo, T., Nakamura, S., Miyamoto, K., Oka, K., Takahashi, M., Akizawa, K., Shibuya, H., Shimizu, C. and Yamaguchi, H. (2016) ‘Acanthamoeba containing endosymbiotic chlamydia isolated from hospital environments and its potential role in inflammatory exacerbation’, *BMC Microbiology*. BioMed Central, 16(1), p. 292. doi: 10.1186/s12866-016-0906-1.

Garate, M., Cao, Z., Bateman, E. and Panjwani, N. (2004) ‘Cloning and characterization of a novel mannose-binding protein of Acanthamoeba.’, *The Journal of biological chemistry*, 279(28), pp. 29849–56. doi: 10.1074/jbc.M402334200.

Garate, M., Cubillos, I., Marchant, J. and Panjwani, N. (2005) ‘Biochemical characterization and functional studies of Acanthamoeba mannose-binding protein.’, *Infection and immunity*, 73(9), pp. 5775–81. doi: 10.1128/IAI.73.9.5775-5781.2005.

Garcia, T. A., Ventura, C. L., Smith, M. A., Merrell, D. S. and O’Brien, A. D. (2013) ‘Cytotoxic necrotizing factor 1 and hemolysin from uropathogenic Escherichia coli elicit different host responses in the murine bladder.’, *Infection and immunity*, 81(1), pp. 99–109. doi: 10.1128/IAI.00605-12.

Garofalo, C. K., Hooton, T. M., Martin, S. M., Stamm, W. E., Palermo, J. J., Gordon, J. I. and Hultgren, S. J. (2007) ‘Escherichia coli from Urine of Female Patients with Urinary Tract Infections Is Competent for Intracellular Bacterial Community Formation’, *Infection and Immunity*, 75(1), pp. 52–60. doi: 10.1128/IAI.01123-06.

Geerlings, S. E., Meiland, R. and Hoepelman, A. I. M. (2002) 'Pathogenesis of bacteriuria in women with diabetes mellitus.', *International journal of antimicrobial agents*, 19(6), pp. 539–45. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12135845 (Accessed: 23 May 2015).

Ghuysen, J. M. (1991) 'Serine Beta-Lactamases and Penicillin-Binding Proteins', *Annual Review of Microbiology*, 45(1), pp. 37–67. doi: 10.1146/annurev.mi.45.100191.000345.

Giske, C. G., Sundsfjord, A. S., Kahlmeter, G., Woodford, N., Nordmann, P., Paterson, D. L., Canton, R. and Walsh, T. R. (2009) 'Redefining extended-spectrum -lactamases: balancing science and clinical need', *Journal of Antimicrobial Chemotherapy*, 63(1), pp. 1–4. doi: 10.1093/jac/dkn444.

Glover, M., Moreira, C. G., Sperandio, V. and Zimmern, P. (2014) 'Recurrent urinary tract infections in healthy and nonpregnant women', *Urological Science*, 25(1), pp. 1–8. doi: 10.1016/j.urols.2013.11.007.

Gomes, T. D. S., Magnet, A., Izquierdo, F., Vaccaro, L., Redondo, F., Bueno, S., Sánchez, M. L., Angulo, S., Fenoy, S., Hurtado, C. and Del Aguila, C. (2016) 'Acanthamoeba spp. in Contact Lenses from Healthy Individuals from Madrid, Spain.', *PloS one*. Public Library of Science, 11(4), p. e0154246. doi: 10.1371/journal.pone.0154246.

Gomes, T. S., Gjiknuri, J., Magnet, A., Vaccaro, L., Ollero, D., Izquierdo, F., Fenoy, S., Hurtado, C. and del Águila, C. (2018) 'The Influence of Acanthamoeba–Legionella Interaction in the Virulence of Two Different Legionella Species', *Frontiers in Microbiology*. Frontiers, 9, p. 2962. doi: 10.3389/fmicb.2018.02962.

González-Robles, A., Omaña-Molina, M., Salazar-Villatoro, L., Flores-Maldonado, C., Lorenzo-Morales, J., Reyes-Batlle, M., Arnalich-Montiel, F. and Martínez-Palomo, A. (2017)

‘Acanthamoeba culbertsoni isolated from a clinical case with intraocular dissemination: Structure and in vitro analysis of the interaction with hamster cornea and MDCK epithelial cell monolayers’, *Experimental Parasitology*, 183, pp. 245–253. doi: 10.1016/j.exppara.2017.09.018.

Gottenbos, B., Busscher, H. J., van der Mei, H. C. and Nieuwenhuis, P. (2002) ‘Pathogenesis and prevention of biomaterial centered infections’, *Journal of Materials Science: Materials in Medicine*. Kluwer Academic Publishers, 13(8), pp. 717–722. doi: 10.1023/A:1016175502756.

Goy, G., Thomas, V., Rimann, K., Jaton, K., Prod’hom, G. and Greub, G. (2007) ‘The Neff strain of Acanthamoeba castellanii, a tool for testing the virulence of Mycobacterium kansasii’, *Research in Microbiology*, 158(4), pp. 393–397. doi: 10.1016/j.resmic.2007.01.003.

Griebling, T. L. (2005) ‘Urologic diseases in america project: trends in resource use for urinary tract infections in men.’, *The Journal of urology*, 173(4), pp. 1288–1294. doi: 10.1097/01.ju.0000155596.98780.82.

Gromova, I. and Celis, J. E. (2006) ‘Protein Detection in Gels by Silver Staining: A Procedure Compatible with Mass Spectrometry’, *Cell Biology, Four-Volume Set*, 4, pp. 219–223. doi: 10.1016/B978-012164730-8/50212-4.

Guicciardi, M. E., Malhi, H., Mott, J. L. and Gores, G. J. (2013) ‘Apoptosis and Necrosis in the Liver’, in *Comprehensive Physiology*. Hoboken, NJ, USA: John Wiley & Sons, Inc., pp. 977–1010. doi: 10.1002/cphy.c120020.

Guimaraes, A. J., Gomes, K. X., Cortines, J. R., Peralta, J. M. and Peralta, R. H. S. (2016) ‘Acanthamoeba spp. as a universal host for pathogenic microorganisms: One bridge from

environment to host virulence', *Microbiological Research*, 193, pp. 30–38. doi: 10.1016/j.micres.2016.08.001.

Guyer, D. M., Henderson, I. R., Nataro, J. P. and Mobley, H. L. (2000) 'Identification of sat, an autotransporter toxin produced by uropathogenic Escherichia coli.', *Molecular microbiology*, 38(1), pp. 53–66. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11029690 (Accessed: 31 December 2018).

Hall, B. G. and Barlow, M. (2005) 'Revised Ambler classification of β-lactamases', *Journal of Antimicrobial Chemotherapy*. Oxford University Press, 55(6), pp. 1050–1051. doi: 10.1093/jac/dki130.

Hall, L. M., Livermore, D. M., Gur, D., Akova, M. and Akalin, H. E. (1993) 'OXA-11, an extended-spectrum variant of OXA-10 (PSE-2) beta-lactamase from Pseudomonas aeruginosa.', *Antimicrobial agents and chemotherapy*, 37(8), pp. 1637–44. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=188033&tool=pmcentrez&rendertype=abstract (Accessed: 22 June 2015).

Hannan, T. J., Totsika, M., Mansfield, K. J., Moore, K. H., Schembri, M. A. and Hultgren, S. J. (2012) 'Host-pathogen checkpoints and population bottlenecks in persistent and intracellular uropathogenic Escherichia coli bladder infection', *FEMS Microbiology Reviews*, 36(3), pp. 616–648. doi: 10.1111/j.1574-6976.2012.00339.x.

Hansen, D. S., Schumacher, H., Hansen, F., Stegger, M., Hertz, F. B., Schønning, K., Justesen, U. S., Frimodt-Møller, N. and DANRES Study Group (2012) 'Extended-spectrum β-lactamase (ESBL) in Danish clinical isolates of Escherichia coli and Klebsiella pneumoniae: Prevalence, β-lactamase distribution, phylogroups, and co-resistance', *Scandinavian Journal of Infectious Diseases*, 44(3), pp. 174–181. doi: 10.3109/00365548.2011.632642.

Hansen, F., Johansen, H. K., Østergaard, C., Arpi, M., Hansen, D. S., Littauer, P., Holm, A., Heltberg, O., Schumacher, H., Fuursted, K., Lykke, M.-A. D., Tønning, B., Hammerum, A. M. and Justesen, U. S. (2014) 'Characterization of Carbapenem Nonsusceptible *Pseudomonas aeruginosa* in Denmark: A Nationwide, Prospective Study', *Microbial Drug Resistance*, 20(1), pp. 22–29. doi: 10.1089/mdr.2013.0085.

Harrison, M. J., Tang, Y. H. and Dowhan, D. H. (2010) 'Protein arginine methyltransferase 6 regulates multiple aspects of gene expression', *Nucleic Acids Research*, 38(7), pp. 2201–2216. doi: 10.1093/nar/gkp1203.

Haslinger-Loffler, 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(8), pp. 1087–1097. doi: 10.1111/j.1462-5822.2005.00533.x.

Heijenoort, van J. (2011) 'Peptidoglycan Hydrolases of Escherichia coli', *Microbiology and Molecular Biology Reviews*, 75(4), pp. 636–663. doi: 10.1128/MMBR.00022-11.

Heimer, S. R., Rasko, D. A., Lockatell, C. V., Johnson, D. E. and Mobley, H. L. T. (2004) 'Autotransporter genes pic and tsh are associated with Escherichia coli strains that cause acute pyelonephritis and are expressed during urinary tract infection.', *Infection and immunity*, 72(1), pp. 593–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/14688142 (Accessed: 1 January 2019).

Henderson, I. R., Czeczulin, J., Eslava, C., Noriega, F. and Nataro, J. P. (1999) 'Characterization of pic, a secreted protease of Shigella flexneri and enteroaggregative Escherichia coli.', *Infection and immunity*. American Society for Microbiology (ASM), 67(11), pp. 5587–96. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10531204 (Accessed: 1 January 2019).

Van der Henst, C., Scrignari, T., Maclachlan, C. and Blokesch, M. (2016) ‘An intracellular replication niche for Vibrio cholerae in the amoeba Acanthamoeba castellanii’, *The ISME Journal*, 10(4), pp. 897–910. doi: 10.1038/ismej.2015.165.

Hershberger, C. and Binkley, S. (1968) ‘Chemistry and metabolism of 3-deoxy-D-mannooctulosonic acid. I. Stereochemical determination’, *The Journal of Biological Chemistry*, 243(7), pp. 1578–1585. doi: 10.1016/j.freeradbiomed.2013.02.024.Microbial.

Hickling, D. R., Sun, T.-T. and Wu, X.-R. (2015) ‘Anatomy and Physiology of the Urinary Tract: Relation to Host Defense and Microbial Infection’, *Microbiology Spectrum*, 3(4). doi: 10.1128/microbiolspec.UTI-0016-2012.

Hilbert, D. W. (2013) *Uropathogenic Escherichia coli: The Pre-Eminent Urinary Tract Infection Pathogen*.

Ho, M., Mettouchi, A., Wilson, B. A. and Lemichez, E. (2018) ‘CNF1-like deamidase domains: common Lego bricks among cancer-promoting immunomodulatory bacterial virulence factors’, *Pathogens and Disease*, 76(5). doi: 10.1093/femspd/fty045.

Ho, P. L., Kurtova, A. and Chan, K. S. (2012) ‘Normal and neoplastic urothelial stem cells: getting to the root of the problem’, *Nature Reviews Urology*, 9(10), pp. 583–594. doi: 10.1038/nrurol.2012.142.

Hooton, T. M., Roberts, P. L. and Stamm, W. E. (1994) ‘Effects of recent sexual activity and use of a diaphragm on the vaginal microflora.’, *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*, 19(2), pp. 274–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/7986899 (Accessed: 23 May 2015).

Hooton, T. M., Scholes, D., Hughes, J. P., Winter, C., Roberts, P. L., Stapleton, A. E., Stergachis, A. and Stamm, W. E. (1996) ‘A prospective study of risk factors for symptomatic urinary

tract infection in young women.', *The New England journal of medicine*, 335(7), pp. 468–74. doi: 10.1056/NEJM199608153350703.

Horner, C., Fawley, W., Morris, K., Parnell, P., Denton, M. and Wilcox, M. (2014) 'Escherichia coli bacteraemia: 2 years of prospective regional surveillance (2010-12)', *Journal of Antimicrobial Chemotherapy*, 69(1), pp. 91–100. doi: 10.1093/jac/dkt333.

Hsu, T., Hutto, D. L., Minion, F. C., Zuerner, R. L. and Wannemuehler, M. J. (2001) 'Cloning of a beta-hemolysin gene of Brachyspira (Serpulina) hyodysenteriae and its expression in Escherichia coli.', *Infection and immunity*, 69(2), pp. 706–11. doi: 10.1128/IAI.69.2.706-711.2001.

Huang, S. H., Chen, Y. H., Fu, Q., Stins, M., Wang, Y., Wass, C. and Kim, K. S. (1999) 'Identification and characterization of an Escherichia coli invasion gene locus, ibeB, required for penetration of brain microvascular endothelial cells.', *Infection and immunity*, 67(5), pp. 2103–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10225861 (Accessed: 1 January 2019).

Huemer, H. P., Eigentler, A., Aschbacher, R. and Larcher, C. (2011) 'Dominance of CTX-M group 1 beta-lactamase enzymes in ESBL producing E. coli from outpatient urines in neighboring regions of Austria and Italy', *Wiener klinische Wochenschrift*, 123(1–2), pp. 41–44. doi: 10.1007/s00508-010-1527-6.

Hughes, R. and Kilvington, S. (2001) 'Comparison of hydrogen peroxide contact lens disinfection systems and solutions against Acanthamoeba polyphaga.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 45(7), pp. 2038–43. doi: 10.1128/AAC.45.7.2038-2043.2001.

Hujer, A. M., Keslar, K. S., Dietenberger, N. J., Bethel, C. R., Endimiani, A. and Bonomo, R. A.

(2009) ‘Detection of SHV beta-lactamases in Gram-negative bacilli using fluorescein-labeled antibodies.’, *BMC microbiology*, 9(1), p. 46. doi: 10.1186/1471-2180-9-46.

Hundt, M. J. and Ruffolo, C. G. (2005) ‘Interaction of Pasteurella multocida with Free-Living Amoebae’, *Applied and Environmental Microbiology*, 71(9), pp. 5458–5464. doi: 10.1128/AEM.71.9.5458-5464.2005.

Hurt, A., Coleman, N. J., Tüzüner, T., Bagis, B., Korkmaz, F. M. and Nicholson, J. W. (2016) ‘Release of cetyl pyridinium chloride from fatty acid chelate temporary dental cement’, *Acta Biomaterialia Odontologica Scandinavica*. Taylor & Francis, 2(1), pp. 1–6. doi: 10.3109/23337931.2015.1125296.

Huws, S. A., Brown, M. R. W. and Smith, A. W. (2005) ‘Interactions of Acanthamoeba polyphaga with Listeria monocytogenes and MRSA’, *The Journal of Eukaryotic Microbiology*. John Wiley & Sons, Ltd (10.1111), 52(2), p. 27S–28S. doi: 10.1111/j.1550-7408.2005.05202003_2_2.x.

Huys, I., Dyason, K., Waelkens, E., Verdonck, F., van Zyl, J., du Plessis, J., Müller, G. J., van der Walt, J., Clynen, E., Schoofs, L. and Tytgat, J. (2002) ‘Purification, characterization and biosynthesis of parabutoxin 3, a component of Parabuthus transvaalicus venom.’, *European journal of biochemistry*, 269(7), pp. 1854–65. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11952787 (Accessed: 30 December 2018).

Hwang, Y. Y., Ramalingam, K., Bienek, D. R., Lee, V., You, T. and Alvarez, R. (2013) ‘Antimicrobial Activity of Nanoemulsion in Combination with Cetylpyridinium Chloride in Multidrug-Resistant Acinetobacter baumannii’, *Antimicrobial Agents and Chemotherapy*, 57(8), pp. 3568–3575. doi: 10.1128/AAC.02109-12.

Ibrahim, Y. W., Boase, D. L. and Cree, I. A. (2009) ‘How Could Contact Lens Wearers Be at

Risk of Acanthamoeba Infection? A Review', *Journal of Optometry*. Elsevier, 2(2), pp. 60–66. doi: 10.3921/joptom.2009.60.

Imai, H., Kita, F., Ikesugi, S., Abe, M., Sogabe, S., Nishimura-Danjobara, Y., Miura, H. and Oyama, Y. (2017) 'Cetylpyridinium chloride at sublethal levels increases the susceptibility of rat thymic lymphocytes to oxidative stress', *Chemosphere*, 170, pp. 118–123. doi: 10.1016/j.chemosphere.2016.12.023.

Imbertbouyer, S., Merlaud, A., Imbert, C., Daniault, G. and Rodier, M. (2004) 'A mannose binding protein is involved in the adherence of species to inert surfaces', *FEMS Microbiology Letters*, 238(1), pp. 207–211. doi: 10.1016/j.femsle.2004.07.036.

Ioannou, C. J., Hanlon, G. W. and Denyer, S. P. (2007) 'Action of Disinfectant Quaternary Ammonium Compounds against Staphylococcus aureus', *Antimicrobial Agents and Chemotherapy*, 51(1), pp. 296–306. doi: 10.1128/AAC.00375-06.

Iovieno, A., Ledee, D. R., Miller, D. and Alfonso, E. C. (2010) 'Detection of Bacterial Endosymbionts in Clinical Acanthamoeba Isolates', *Ophthalmology*, 117(3), p. 445–452.e3. doi: 10.1016/j.ophtha.2009.08.033.

Iovieno, A., Miller, D., Lonnen, J., Kilvington, S. and Alfonso, E. C. (2011) 'Extraction of Acanthamoeba DNA by use of Chelex resin.', *Journal of clinical microbiology*. American Society for Microbiology, 49(1), pp. 476–7. doi: 10.1128/JCM.01795-10.

Iqbal, J., Siddiqui, R. and Khan, N. A. (2014) 'Acanthamoeba and bacteria produce antimicrobials to target their counterpart.', *Parasites & vectors*, 7, p. 56. doi: 10.1186/1756-3305-7-56.

Iredell, J., Brown, J. and Tagg, K. (2016) 'Antibiotic resistance in Enterobacteriaceae: mechanisms and clinical implications', *BMJ*, p. h6420. doi: 10.1136/bmj.h6420.

Jacoby, G. A. (2009) ‘AmpC beta-lactamases.’, *Clinical microbiology reviews*, 22(1), p. 161–82, Table of Contents. doi: 10.1128/CMR.00036-08.

Jafari, A., Aslani, M. M. and Bouzari, S. (2012) ‘Escherichia coli: a brief review of diarrheagenic pathotypes and their role in diarrheal diseases in Iran.’, *Iranian journal of microbiology*, 4(3), pp. 102–17. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3465535&tool=pmcentrez&rendertype=abstract (Accessed: 1 November 2016).

Jamal, W. Y., Albert, M. J. and Rotimi, V. O. (2016) ‘High Prevalence of New Delhi Metallo-β-Lactamase-1 (NDM-1) Producers among Carbapenem-Resistant Enterobacteriaceae in Kuwait’, *PLOS ONE*. Edited by M. Galdiero. Public Library of Science, 11(3), p. e0152638. doi: 10.1371/journal.pone.0152638.

Jaurin, B. and Grundström, T. (1981) ‘ampC cephalosporinase of Escherichia coli K-12 has a different evolutionary origin from that of beta-lactamases of the penicillinase type.’, *Proceedings of the National Academy of Sciences of the United States of America*, 78(8), pp. 4897–901. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6795623 (Accessed: 12 December 2018).

Jean, S.-S. and Hsueh, P.-R. (2011) ‘High burden of antimicrobial resistance in Asia’, *International Journal of Antimicrobial Agents*, 37(4), pp. 291–295. doi: 10.1016/j.ijantimicag.2011.01.009.

Jeon, K. W. (1995) ‘Bacterial endosymbiosis in amoebae’, *Trends in Cell Biology*. Elsevier Current Trends, 5(3), pp. 137–140. doi: 10.1016/S0962-8924(00)88966-7.

Jeong, H. J., Jang, E. S., Han, B. I., Lee, K. H., Ock, M. S., Kong, H. H., Chung, D. Il, Seol, S. Y., Cho, D. T. and Yu, H. S. (2007) ‘Acanthamoeba: Could it be an environmental host of

Shigella?', *Experimental Parasitology*, 115(2), pp. 181–186. doi: 10.1016/j.exppara.2006.08.002.

Jeong, H. J. and Yu, H. S. (2005) 'The role of domestic tap water in Acanthamoeba contamination in contact lens storage cases in Korea.', *The Korean journal of parasitology*, 43(2), pp. 47–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/15951638 (Accessed: 13 December 2018).

Jiao, Y., Niu, L., Ma, S., Li, J., Tay, F. R. and Chen, J. (2017) 'Quaternary ammonium-based biomedical materials: State-of-the-art, toxicological aspects and antimicrobial resistance', *Progress in Polymer Science*. Pergamon, 71, pp. 53–90. doi: 10.1016/J.PROGPOLYMSCI.2017.03.001.

Johnson, J. R. (1991) 'Virulence factors in Escherichia coli urinary tract infection.', *Clinical microbiology reviews*, 4(1), pp. 80–128. doi: 10.1128/CMR.4.1.80.

Johnson, J. R., Johnston, B., Kuskowski, M. A., Nougayrede, J.-P. and Oswald, E. (2008) 'Molecular Epidemiology and Phylogenetic Distribution of the Escherichia coli pks Genomic Island', *Journal of Clinical Microbiology*, 46(12), pp. 3906–3911. doi: 10.1128/JCM.00949-08.

Johnson, J. R., Kuskowski, M. A., Gajewski, A., Soto, S., Horcajada, J. P., Jimenez de Anta, M. T. and Vila, J. (2005) 'Extended Virulence Genotypes and Phylogenetic Background of *Escherichia coli* Isolates from Patients with Cystitis, Pyelonephritis, or Prostatitis', *The Journal of Infectious Diseases*, 191(1), pp. 46–50. doi: 10.1086/426450.

Johnson, J. R., Menard, M., Johnston, B., Kuskowski, M. A., Nichol, K. and Zhanel, G. G. (2009) 'Epidemic clonal groups of Escherichia coli as a cause of antimicrobial-resistant urinary tract infections in Canada, 2002 to 2004.', *Antimicrobial agents and chemotherapy*.

American Society for Microbiology (ASM), 53(7), pp. 2733–9. doi: 10.1128/AAC.00297-09.

Johnson, J. R. and Stell, A. L. (2000) 'Extended Virulence Genotypes of *Escherichia coli* Strains from Patients with Urosepsis in Relation to Phylogeny and Host Compromise', *The Journal of Infectious Diseases*. Oxford University Press, 181(1), pp. 261–272. doi: 10.1086/315217.

Johnson, J. R., Tchesnokova, V., Johnston, B., Clabots, C., Roberts, P. L., Billig, M., Riddell, K., Rogers, P., Qin, X., Butler-Wu, S., Price, L. B., Aziz, M., Nicolas-Chanoine, M.-H., DebRoy, C., Robicsek, A., Hansen, G., Urban, C., Platell, J., Trott, D. J., Zhanel, G., Weissman, S. J., Cookson, B. T., Fang, F. C., Limaye, A. P., Scholes, D., Chattopadhyay, S., Hooper, D. C. and Sokurenko, E. V. (2013) 'Abrupt Emergence of a Single Dominant Multidrug-Resistant Strain of Escherichia coli', *The Journal of Infectious Diseases*, 207(6), pp. 919–928. doi: 10.1093/infdis/jis933.

Johnson, T. J., Logue, C. M., Johnson, J. R., Kuskowski, M. A., Sherwood, J. S., Barnes, H. J., DebRoy, C., Wannemuehler, Y. M., Obata-Yasuoka, M., Spanjaard, L. and Nolan, L. K. (2012) 'Associations Between Multidrug Resistance, Plasmid Content, and Virulence Potential Among Extraintestinal Pathogenic and Commensal *Escherichia coli* from Humans and Poultry', *Foodborne Pathogens and Disease*, 9(1), pp. 37–46. doi: 10.1089/fpd.2011.0961.

Johnson, T. J., Siek, K. E., Johnson, S. J. and Nolan, L. K. (2006) 'DNA sequence of a ColV plasmid and prevalence of selected plasmid-encoded virulence genes among avian Escherichia coli strains.', *Journal of bacteriology*. American Society for Microbiology (ASM), 188(2), pp. 745–58. doi: 10.1128/JB.188.2.745-758.2006.

De Jonckheere, J. F. (1991) 'Ecology of Acanthamoeba', *Clinical Infectious Diseases*, 13(Supplement 5), pp. S385–S387. doi: 10.1093/clind/13.Supplement_5.S385.

Juhas, M. (2015) 'Horizontal gene transfer in human pathogens.', *Critical reviews in microbiology*, 41(1), pp. 101–8. doi: 10.3109/1040841X.2013.804031.

Justice, S. S., Hung, C., Theriot, J. A., Fletcher, D. A., Anderson, G. G., Footer, M. J. and Hultgren, S. J. (2004) 'Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract pathogenesis', *Proceedings of the National Academy of Sciences*, 101(5), pp. 1333–1338. doi: 10.1073/pnas.0308125100.

Kalpoe, J. S., Al Naiemi, N., Poirel, L. and Nordmann, P. (2011) 'Detection of an Ambler class D OXA-48-type -lactamase in a Klebsiella pneumoniae strain in The Netherlands', *Journal of Medical Microbiology*, 60(5), pp. 677–678. doi: 10.1099/jmm.0.028308-0.

Kamolvit, W., Sidjabat, H. E. and Paterson, D. L. (2015) 'Molecular Epidemiology and Mechanisms of Carbapenem Resistance of *Acinetobacter* spp. in Asia and Oceania', *Microbial Drug Resistance*, 21(4), pp. 424–434. doi: 10.1089/mdr.2014.0234.

Kang, A.-Y., Park, A.-Y., Shin, H.-J., Khan, N. A., Maciver, S. K. and Jung, S.-Y. (2018) 'Production of a monoclonal antibody against a mannose-binding protein of Acanthamoeba culbertsoni and its localization', *Experimental Parasitology*, 192, pp. 19–24. doi: 10.1016/j.exppara.2018.07.009.

Kang, C.-I., Song, J.-H., Chung, D. R., Peck, K. R., Ko, K. S., Yeom, J.-S., Ki, H. K., Son, J. S., Lee, S. S., Kim, Y.-S., Jung, S.-I., Kim, S.-W., Chang, H.-H., Ryu, S. Y., Kwon, K. T., Lee, H., Moon, C., Shin, S. Y. and Korean Network for Study of Infectious Diseases (KONSID) (2010) 'Risk factors and treatment outcomes of community-onset bacteraemia caused by extended-spectrum β-lactamase-producing Escherichia coli', *International Journal of Antimicrobial Agents*, 36(3), pp. 284–287. doi: 10.1016/j.ijantimicag.2010.05.009.

Karam, M. R. A., Habibi, M. and Bouzari, S. (2018) 'Relationships between Virulence Factors

and Antimicrobial Resistance among Escherichia coli Isolated from Urinary Tract Infections and Commensal Isolates in Tehran, Iran.', *Osong public health and research perspectives*. Korea Centers for Disease Control and Prevention, 9(5), pp. 217–224. doi: 10.24171/j.phrp.2018.9.5.02.

Karamati, S. A., Niyyati, M., Lorenzo-Morales, J. and Lasjerdi, Z. (2016) 'Isolation and molecular characterization of Acanthamoeba genotypes isolated from soil sources of public and recreational areas in Iran', *Acta Parasitologica*, 61(4), pp. 784–789. doi: 10.1515/ap-2016-0108.

Karaś, M., Turska-Szewczuk, A., Marczak, M., Jaszek, M., Janczarek, M., Dworaczek, K., Stefaniuk, D. and Wydrych, J. (2018) 'A Mutation in the Mesorhizobium loti oatB Gene Alters the Physicochemical Properties of the Bacterial Cell Wall and Reduces Survival inside Acanthamoeba castellanii', *International Journal of Molecular Sciences*, 19(11), p. 3510. doi: 10.3390/ijms19113510.

Kaushal, V., Chhina, D. K., Kumar, R., Pannu, H. S., Dhooria, H. P. S. and Chhina, R. S. (2008) 'Acanthamoeba encephalitis.', *Indian journal of medical microbiology*. Medknow Publications, 26(2), pp. 182–4. doi: 10.4103/0255-0857.40539.

Kavaler, E. (2007) *A Seat on the Aisle, Please!: The Essential Guide to Urinary Tract Problems in Women*. Springer Science & Business Media. Available at: https://books.google.co.uk/books/about/A_Seat_on_the_Aisle_Please.html?id=_0IAIZZiH_MC&pgis=1 (Accessed: 3 April 2016).

Kawamura-Sato, K., Yoshida, R., Shibayama, K. and Ohta, M. (2010) 'Virulence genes, quinolone and fluoroquinolone resistance, and phylogenetic background of uropathogenic Escherichia coli strains isolated in Japan.', *Japanese journal of infectious diseases*, 63(2), pp. 113–5. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20332573 (Accessed: 23

December 2018).

Keat, T. (2011) *CHARACTERISATION OF ESBL-PRODUCING ENTEROBACTERIACEAE OF FOUR LOCAL VEGETABLES TOH WAI KEAT BACHELOR OF SCIENCE (HONS) BIOTECHNOLOGY FACULTY OF SCIENCE UNIVERSITI TUNKU ABDUL RAHMAN*.

Kelley, S. P., Courtneidge, H. R., Birch, R. E., Contreras-Sanz, A., Kelly, M. C., Durodie, J., Peppiatt-Wildman, C. M., Farmer, C. K., Delaney, M. P., Malone-Lee, J., Harber, M. A. and Wildman, S. S. (2014) 'Urinary ATP and visualization of intracellular bacteria: a superior diagnostic marker for recurrent UTI in renal transplant recipients?', *SpringerPlus*, 3, p. 200. doi: 10.1186/2193-1801-3-200.

Khan, N. A. (2006) 'Acanthamoeba: Biology and increasing importance in human health', *FEMS Microbiology Reviews*. Oxford University Press, 30(4), pp. 564–595. doi: 10.1111/j.1574-6976.2006.00023.x.

Khan, N. A. (2008) *Emerging protozoan pathogens*. Taylor & Francis Group. Available at: https://www.crcpress.com/Emerging-Protozoan-Pathogens/Khan/p/book/9780415428644 (Accessed: 15 December 2018).

Khan, N. A. (2009) *Acanthamoeba: Biology and Pathogenesis*. Horizon Scientific Press. Available at: https://books.google.com/books?id=lXfp-1SOP3cC&pgis=1 (Accessed: 23 June 2015).

Khan, N. A., Jarroll, E. L., Panjwani, N., Cao, Z. and Paget, T. A. (2000) 'Proteases as markers for differentiation of pathogenic and nonpathogenic species of Acanthamoeba.', *Journal of clinical microbiology*. American Society for Microbiology (ASM), 38(8), pp. 2858–61. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10921939 (Accessed: 13 December 2018).

Khan, N. A. and Paget, T. A. (2002) ‘Molecular Tools for Speciation and Epidemiological Studies of Acanthamoeba’, *Current Microbiology*, 44(6), pp. 444–449. doi: 10.1007/s00284-001-0050-4.

Kim, Y. R., Lee, S. E., Kook, H., Yeom, J. A., Na, H. S., Kim, S. Y., Chung, S. S., Choy, H. E. and Rhee, J. H. (2008) ‘Vibrio vulnificus RTX toxin kills host cells only after contact of the bacteria with host cells’, *Cellular Microbiology*, 10(4), pp. 848–862. doi: 10.1111/j.1462-5822.2007.01088.x.

Kisiela, D. I., Avagyan, H., Friend, D., Jalan, A., Gupta, S., Interlandi, G., Liu, Y., Tchesnokova, V., Rodriguez, V. B., Sumida, J. P., Strong, R. K., Wu, X.-R., Thomas, W. E. and Sokurenko, E. V (2015) ‘Inhibition and Reversal of Microbial Attachment by an Antibody with Parasteric Activity against the FimH Adhesin of Uropathogenic E. coli.’, *PLoS pathogens*, 11(5), p. e1004857. doi: 10.1371/journal.ppat.1004857.

Klein, G., Müller-Loennies, S., Lindner, B., Kobylak, N., Brade, H. and Raina, S. (2013) ‘Molecular and structural basis of inner core lipopolysaccharide alterations in escherichia coli: Incorporation of glucuronic acid and phosphoethanolamine in the heptose region’, *Journal of Biological Chemistry*, 288(12), pp. 8111–8127. doi: 10.1074/jbc.M112.445981.

Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M. and Wiedemann, B. (1985) ‘Evolution of plasmid-coded resistance to broad-spectrum cephalosporins.’, *Antimicrobial agents and chemotherapy*. American Society for Microbiology Journals, 28(2), pp. 302–7. doi: 10.1128/AAC.28.2.302.

Klose, J. (1975) ‘Protein mapping by combined isoelectric focusing and electrophoresis of mouse tissues. A novel approach to testing for induced point mutations in mammals.’, *Humangenetik*, 26(3), pp. 231–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1093965 (Accessed: 30 December 2018).

Kojima, S. and Nikaido, H. (2013) 'Permeation rates of penicillins indicate that Escherichia coli porins function principally as nonspecific channels', *Proceedings of the National Academy of Sciences*, 110(28), pp. E2629–E2634. doi: 10.1073/pnas.1310333110.

Kolar, M., Bardon, J., Chroma, M., Hricova, K., Stosova, T., Sauer, P. and Koukalova, D. (2010) 'ESBL and AmpC beta-lactamase-producing Enterobacteriaceae in poultry in the Czech Republic', *Veterinarni Medicina*, 55(3), pp. 119–124.

Koturbash, I., Thomas, J. E., Kovalchuk, O. and Kovalchuk, I. (2009) 'Heat-killed bacteria induce genome instability in mouse small intestine, liver and spleen tissues', *Cell Cycle*, 8(12), pp. 1935–1939. doi: 10.4161/cc.8.12.8797.

Kouokam, J. C., Wai, S. N., Fällman, M., Dobrindt, U., Hacker, J. and Uhlin, B. E. (2006) 'Active cytotoxic necrotizing factor 1 associated with outer membrane vesicles from uropathogenic Escherichia coli.', *Infection and immunity*, 74(4), pp. 2022–30. doi: 10.1128/IAI.74.4.2022-2030.2006.

Kourai, H., Yabuhara, T., Shirai, A., Maeda, T. and Nagamune, H. (2006) 'Syntheses and antimicrobial activities of a series of new bis-quaternary ammonium compounds', *European Journal of Medicinal Chemistry*, 41(4), pp. 437–444. doi: 10.1016/j.ejmech.2005.10.021.

Kranzer, K., Ahmed, N., Hoffman, P. and Pink, F. (2016) *Protocol for Environmental Sampling, Processing and Culturing of Water and Air Samples for the Isolation of Slow-Growing Mycobacteria: Standard operating procedure*. Available at: www.gov.uk/phe (Accessed: 30 December 2018).

Kreuzer, K., Adamczyk, J., Iijima, M., Wagner, M., Scheu, S. and Bonkowski, M. (2006) 'Grazing of a common species of soil protozoa (Acanthamoeba castellanii) affects

rhizosphere bacterial community composition and root architecture of rice (Oryza sativa L.)', *Soil Biology and Biochemistry*. Pergamon, 38(7), pp. 1665–1672. doi: 10.1016/J.SOILBIO.2005.11.027.

Kuburich, N. A., Adhikari, N. and Hadwiger, J. A. (2016) 'Acanthamoeba and Dictyostelium Use Different Foraging Strategies', *Protist*, 167(6), pp. 511–525. doi: 10.1016/j.protis.2016.08.006.

Kucheria, R., Dasgupta, P., Sacks, S. H., Khan, M. S. and Sheerin, N. S. (2005) 'Urinary tract infections: new insights into a common problem.', *Postgraduate medical journal*, 81(952), pp. 83–86. doi: 10.1136/pgmj.2004.023036.

Kulp, A. and Kuehn, M. J. (2010) 'Biological functions and biogenesis of secreted bacterial outer membrane vesicles.', *Annual review of microbiology*, 64, pp. 163–84. doi: 10.1146/annurev.micro.091208.073413.

Kumar, P. S. and Khan, M. W. (2011) 'Res earch Article Community-acquired urinary tract infection in the elderly', 4(1), pp. 1–4.

Kumar, V., Abbas, A. K., Aster, J. C. and Perkins, J. A. (2015) *Robbins and Cotran pathologic basis of disease*. Ninth edition. Elsevier/Saunders,. Available at: http://voyagercatalog.kumc.edu/Record/181593/TOC (Accessed: 12 December 2018).

Kunin, C. M. and McCormack, R. C. (1968) 'An epidemiologic study of bacteriuria and blood pressure among nuns and working women.', *The New England journal of medicine*, 278(12), pp. 635–42. doi: 10.1056/NEJM196803212781201.

Kurazono, H., Yamamoto, S., Nakano, M., Nair, G. B., Terai, A., Chaicumpa, W. and Hayashi, H. (2000) 'Characterization of a putative virulence island in the chromosome of uropathogenic Escherichia coli possessing a gene encoding a uropathogenic-specific

protein', *Microbial Pathogenesis*, 28(3), pp. 183–189. doi: 10.1006/mpat.1999.0331.

Landraud, L., Gauthier, M., Fosse, T. and Boquet, P. (2000) 'Frequency of Escherichia coli strains producing the cytotoxic necrotizing factor (CNF1) in nosocomial urinary tract infections.', *Letters in applied microbiology*, 30(3), pp. 213–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10747253 (Accessed: 27 May 2015).

Ledee, D. R., Booton, G. C., Awwad, M. H., Sharma, S., Aggarwal, R. K., Niszl, I. A., Markus, M. B., Fuerst, P. A. and Byers, T. J. (2003) 'Advantages of using mitochondrial 16S rDNA sequences to classify clinical isolates of Acanthamoeba.', *Investigative ophthalmology & visual science*, 44(3), pp. 1142–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12601042 (Accessed: 13 December 2018).

Lee, S.-M., Lee, D.-I., Park, S.-H., Yu, H.-S., Lee, J.-E. and Lee, J.-S. (2017) 'Effect of Multipurpose Solution Combined With Autophagy Inhibitors on Adhesion of Acanthamoeba trophozoites to Silicone Hydrogel Contact Lenses', *Cornea*, 36(12), pp. 1538–1543. doi: 10.1097/ICO.0000000000001340.

Lewis, D. A., Brown, R., Williams, J., White, P., Jacobson, S. K., Marchesi, J. R. and Drake, M. J. (2013) 'The human urinary microbiome; bacterial DNA in voided urine of asymptomatic adults', *Frontiers in Cellular and Infection Microbiology*, 3, p. 41. doi: 10.3389/fcimb.2013.00041.

Lewis, K. (2013) 'Platforms for antibiotic discovery', *Nature Reviews Drug Discovery*, 12(5), pp. 371–387. doi: 10.1038/nrd3975.

Li, D., Liu, B., Chen, M., Guo, D., Guo, X., Liu, F., Feng, L. and Wang, L. (2010) 'A multiplex PCR method to detect 14 Escherichia coli serogroups associated with urinary tract infections.', *Journal of microbiological methods*, 82(1), pp. 71–7. doi:

10.1016/j.mimet.2010.04.008.

Li, J., Olaleye, E. D., 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*. Elsevier, 87, pp. 64–72. doi: 10.1016/J.POLYMER.2016.01.073.

Li, X.-Z., Plésiat, P. and Nikaido, H. (2015) 'The Challenge of Efflux-Mediated Antibiotic Resistance in Gram-Negative Bacteria', *Clinical Microbiology Reviews*, 28(2), pp. 337–418. doi: 10.1128/CMR.00117-14.

Lillington, J., Geibel, S. and Waksman, G. (2014) 'Biogenesis and adhesion of type 1 and P pili.', *Biochimica et biophysica acta*, 1840(9), pp. 2783–93. doi: 10.1016/j.bbagen.2014.04.021.

Lim, S. P. and Nikaido, H. (2010) 'Kinetic Parameters of Efflux of Penicillins by the Multidrug Efflux Transporter AcrAB-TolC of Escherichia coli', *Antimicrobial Agents and Chemotherapy*, 54(5), pp. 1800–1806. doi: 10.1128/AAC.01714-09.

Liu, Y.-Y., Wang, Y., Walsh, T. R., Yi, L.-X., Zhang, R., Spencer, J., Doi, Y., Tian, G., Dong, B., Huang, X., Yu, L.-F., Gu, D., Ren, H., Chen, X., Lv, L., He, D., Zhou, H., Liang, Z., Liu, J.-H. and Shen, J. (2016) 'Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study', *The Lancet Infectious Diseases*, 16(2), pp. 161–168. doi: 10.1016/S1473-3099(15)00424-7.

Livermore, D. M. and Woodford, N. (2006) 'The β-lactamase threat in Enterobacteriaceae, Pseudomonas and Acinetobacter', *Trends in Microbiology*, 14(9), pp. 413–420. doi: 10.1016/j.tim.2006.07.008.

Lloyd, D. (2014) 'Encystment in Acanthamoeba castellanii: A review', *Experimental*

Parasitology, 145, pp. S20–S27. doi: 10.1016/j.exppara.2014.03.026.

Lo, T. S. and Alonto, A. (2011) 'A Review of Uncomplicated Urinary Tract Infections', *Infection*, pp. 3–15.

Lolis, E. and Bucala, R. (2003) 'Therapeutic approaches to innate immunity: severe sepsis and septic shock.', *Nature reviews. Drug discovery*, 2(8), pp. 635–45. doi: 10.1038/nrd1153.

Lorenzo-Morales, J., Coronado-Alvarez, N., Martínez-Carretero, E., Maciver, S. K. and Valladares, B. (2007) 'Detection of four adenovirus serotypes within water-isolated strains of Acanthamoeba in the Canary Islands, Spain.', *The American journal of tropical medicine and hygiene*, 77(4), pp. 753–6. Available at: http://www.ncbi.nlm.nih.gov/pubmed/17978083 (Accessed: 21 December 2018).

Lorenzo-Morales, J., Khan, N. A. and Walochnik, J. (2015) 'An update on Acanthamoeba keratitis: diagnosis, pathogenesis and treatment.', *Parasite (Paris, France)*. EDP Sciences, 22, p. 10. doi: 10.1051/parasite/2015010.

Lorenzo-Morales, J., Lindo, J. F., Martinez, E., Calder, D., Figueruelo, E., Valladares, B. and Ortega-Rivas, A. (2005) 'Pathogenic *Acanthamoeba* strains from water sources in Jamaica, West Indies', *Annals of Tropical Medicine & Parasitology*, 99(8), pp. 751–758. doi: 10.1179/136485905X65215.

Lorenzo-Morales, J., López-Darias, M., Martínez-Carretero, E. and Valladares, B. (2007) 'Isolation of potentially pathogenic strains of Acanthamoeba in wild squirrels from the Canary Islands and Morocco', *Experimental Parasitology*, 117(1), pp. 74–79. doi: 10.1016/j.exppara.2007.03.014.

Lorenzo-Morales, J., Martín-Navarro, C. M., López-Arencibia, A., Arnalich-Montiel, F., Piñero, J. E. and Valladares, B. (2013) 'Acanthamoeba keratitis: an emerging disease gathering

importance worldwide?', *Trends in Parasitology*, 29(4), pp. 181–187. doi: 10.1016/j.pt.2013.01.006.

Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Mart�nez, E. and Valladares, B. (2005) 'Isolation and identification of pathogenic Acanthamoeba strains in Tenerife, Canary Islands, Spain from water sources', *Parasitology Research*, 95(4), pp. 273–277. doi: 10.1007/s00436-005-1301-2.

Lorenzo-Morales, J., Ortega-Rivas, A., Martínez, E., Khoubbane, M., Artigas, P., Periago, M. V., Foronda, P., Abreu-Acosta, N., Valladares, B. and Mas-Coma, S. (2006) 'Acanthamoeba isolates belonging to T1, T2, T3, T4 and T7 genotypes from environmental freshwater samples in the Nile Delta region, Egypt', *Acta Tropica*. Elsevier, 100(1–2), pp. 63–69. doi: 10.1016/J.ACTATROPICA.2006.09.008.

Lu, Y., Bhushan, S., Tchatalbachev, S., Marconi, M., Bergmann, M., Weidner, W., Chakraborty, T. and Meinhardt, A. (2013) 'Necrosis Is the Dominant Cell Death Pathway in Uropathogenic Escherichia coli Elicited Epididymo-Orchitis and Is Responsible for Damage of Rat Testis', *PLoS ONE*. Edited by S. Schlatt, 8(1), p. e52919. doi: 10.1371/journal.pone.0052919.

Lüthje, P. and Brauner, A. (2016) 'Novel Strategies in the Prevention and Treatment of Urinary Tract Infections', *Pathogens*, 5(1), p. 13. doi: 10.3390/pathogens5010013.

Macey, M. G. (2007) *Flow cytometry: Principles and clinical applications*. Edited by M. G. Macey. Totowa, NJ: Humana Press. doi: 10.1007/978-1-59745-451-3.

Madigan, M., Martinko, J., Dunlap, P. and Clark, D. (2009) *Brock Biology of Microorganisms*. 12th Editi, *Benjamin Cummings Copyright*. 12th Editi. New Jersey.

Mahdi, A. B., Patrick, J. B., Alireza, K., Hadi, H. N. and Amin, S. M. (2015) 'Prevalence and

clonal distribution of avian Escherichia coli isolates harboring increased serum survival (iss) gene', *Journal of Applied Poultry Research*. Oxford University Press, 25(1), pp. 67–73. doi: 10.3382/japr/pfv064.

Mahesh, E., Medha, Y., Indumathi, A., Prithvi, S., Mohammed Wasim, K. and Punith, K. (2011) 'Community-acquired urinary tract infection in the elderly', *British Journal of Medical Practitioners*, 4(1), pp. 1–4.

Mahjoub-Messai, F., Bidet, P., Caro, V., Diancourt, L., Biran, V., Aujard, Y., Bingen, E. and Bonacorsi, S. (2011) 'Escherichia coli Isolates Causing Bacteremia via Gut Translocation and Urinary Tract Infection in Young Infants Exhibit Different Virulence Genotypes', *The Journal of Infectious Diseases*, 203(12), pp. 1844–1849. doi: 10.1093/infdis/jir189.

Maisonneuve, E., Cateau, E., Leveque, N., Kaaki, S., Beby-Defaux, A. and Rodier, M.-H. (2017) 'Acanthamoeba castellanii is not be an adequate model to study human adenovirus interactions with macrophagic cells', *PLOS ONE*. Edited by I. Ulasov, 12(6), p. e0178629. doi: 10.1371/journal.pone.0178629.

Malekzadegan, Y., Khashei, R., Sedigh Ebrahim-Saraie, H. and Jahanabadi, Z. (2018) 'Distribution of virulence genes and their association with antimicrobial resistance among uropathogenic Escherichia coli isolates from Iranian patients', *BMC Infectious Diseases*, 18(1), p. 572. doi: 10.1186/s12879-018-3467-0.

Malloy, A. M. W. and Campos, J. M. (2011) 'Extended-spectrum Beta-lactamases', *The Pediatric Infectious Disease Journal*, 30(12), pp. 1092–1093. doi: 10.1097/INF.0b013e31823c0e9d.

Marciano-Cabral, F. and Cabral, G. (2003) 'Acanthamoeba spp. as Agents of Disease in Humans', *Clinical Microbiology Reviews*, 16(2), pp. 273–307. doi: 10.1128/CMR.16.2.273-

307.2003.

Martin-Navarro, C. M., Lorenzo-Morales, J., Cabrera-Serra, M. G., Rancel, F., Coronado-Alvarez, N. M., Pinero, J. E. and Valladares, B. (2008) 'The potential pathogenicity of chlorhexidine-sensitive Acanthamoeba strains isolated from contact lens cases from asymptomatic individuals in Tenerife, Canary Islands, Spain', *Journal of Medical Microbiology*, 57(11), pp. 1399–1404. doi: 10.1099/jmm.0.2008/003459-0.

Masadeh, M. M., Gharaibeh, S. F., Alzoubi, K. H., Al-Azzam, S. I. and Obeidat, W. M. (2013) 'Antimicrobial Activity of Common Mouthwash Solutions on Multidrug-Resistance Bacterial Biofilms', *Journal of Clinical Medicine Research*, 5(5), pp. 389–94. doi: 10.4021/jocmr1535w.

Maschio, V. J., Chies, F., Carlesso, A. M., Carvalho, A., Rosa, S. P., Van Der Sand, S. T. and Rott, M. B. (2015) 'Acanthamoeba T4, T5 and T11 Isolated From Mineral Water Bottles in Southern Brazil', *Current Microbiology*, 70(1), pp. 6–9. doi: 10.1007/s00284-014-0676-7.

Matin, A. and Jung, S.-Y. (2011) 'Phospholipase activities in clinical and environmental isolates of Acanthamoeba.', *The Korean journal of parasitology*. Korean Society for Parasitology, 49(1), pp. 1–8. doi: 10.3347/kjp.2011.49.1.1.

Matsui, T., Maeda, T., Kusakabe, S., Arita, H., Yagita, K., Morii, E. and Kanakura, Y. (2018) 'A case report of granulomatous amoebic encephalitis by Group 1 Acanthamoeba genotype T18 diagnosed by the combination of morphological examination and genetic analysis.', *Diagnostic pathology*. BioMed Central, 13(1), p. 27. doi: 10.1186/s13000-018-0706-z.

Matsuura, M. (2013) 'Structural Modifications of Bacterial Lipopolysaccharide that Facilitate Gram-Negative Bacteria Evasion of Host Innate Immunity.', *Frontiers in immunology*, 4, p. 109. doi: 10.3389/fimmu.2013.00109.

Mazzariol, A., Bazaj, A. and Cornaglia, G. (2017) 'Multi-drug-resistant Gram-negative bacteria causing urinary tract infections: a review', *Journal of Chemotherapy*, 29(sup1), pp. 2–9. doi: 10.1080/1120009X.2017.1380395.

McIlwain, D. R., Berger, T. and Mak, T. W. (2013) 'Caspase Functions in Cell Death and Disease', *Cold Spring Harbor Perspectives in Biology*, 5(4), pp. a008656–a008656. doi: 10.1101/cshperspect.a008656.

Mehall, L. N., Pohlman, F. W., Brown, A. H., Dias-Morse, P. N., McKenzie, L. M. and Mohan, A. (2015) 'The influence of trisodium phosphate, potassium lactate, sodium metasilicate, cetylpyridinium chloride, or water as antimicrobial intervention systems on microbiological and instrumental color characteristics of beef biceps femoris muscles', *The Professional Animal Scientist*, 31(4), pp. 342–348. doi: 10.15232/pas.2014-01338.

Melin, V. E., Potineni, H., Hunt, P., Griswold, J., Siems, B., Werre, S. R. and Hrubec, T. C. (2014) 'Exposure to common quaternary ammonium disinfectants decreases fertility in mice', *Reproductive Toxicology*, 50, pp. 163–170. doi: 10.1016/j.reprotox.2014.07.071.

Mengue, L., Richard, F.-J., Caubet, Y., Rolland, S., Héchard, Y. and Samba-Louaka, A. (2017) 'Legionella pneumophila decreases velocity of Acanthamoeba castellanii', *Experimental Parasitology*, 183, pp. 124–127. doi: 10.1016/j.exppara.2017.07.013.

Mills, M., Meysick, K. C. and O'Brien, A. D. (2000) 'Cytotoxic necrotizing factor type 1 of uropathogenic Escherichia coli kills cultured human uroepithelial 5637 cells by an apoptotic mechanism.', *Infection and immunity*, 68(10), pp. 5869–80. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=101549&tool=pmcentrez&rendertype=abstract (Accessed: 27 May 2015).

Mitsumori, K., Terai, A., Yamamoto, S., Ishitoya, S. and Yoshida, O. (1999) 'Virulence

Characteristics of *Escherichia coli* in Acute Bacterial Prostatitis', *The Journal of Infectious Diseases*, 180(4), pp. 1378–1381. doi: 10.1086/314976.

Mohamudha, P. R., Harish, B. N. and Parija, S. C. (2012) 'Molecular description of plasmid-mediated AmpC ??-lactamases among nosocomial isolates of Escherichia coli & Klebsiella pneumoniae from six different hospitals in India', *Indian Journal of Medical Research*, 135(1), pp. 114–119. doi: 10.4103/0971-5916.93433.

Molmeret, M., Bitar, D. M., Han, L. and Kwaik, Y. A. (2004) 'Disruption of the Phagosomal Membrane and Egress of Legionella pneumophila into the Cytoplasm during the Last Stages of Intracellular Infection of Macrophages and Acanthamoeba polyphaga', *Infection and Immunity*, 72(7), pp. 4040–4051. doi: 10.1128/IAI.72.7.4040-4051.2004.

Moon, E.-K., Xuan, Y.-H., Chung, D.-I., Hong, Y. and Kong, H.-H. (2011) 'Microarray analysis of differentially expressed genes between cysts and trophozoites of Acanthamoeba castellanii.', *The Korean journal of parasitology*, 49(4), pp. 341–7. doi: 10.3347/kjp.2011.49.4.341.

Mora-Rillo, M., Fernández-Romero, N., Francisco, C. N.-S., Díez-Sebastián, J., Romero-Gómez, M. P., Fernández, F. A., López, J. R. A. and Mingorance, J. (2015) 'Impact of virulence genes on sepsis severity and survival in Escherichia coli bacteremia.', *Virulence*. Taylor & Francis, 6(1), pp. 93–100. doi: 10.4161/21505594.2014.991234.

Mora-Rillo, M., Fernández-Romero, N., Navarro-San Francisco, C., Díez-Sebastián, J., Romero-Gómez, M. P., Arnalich Fernández, F., Arribas López, J. R. and Mingorance, J. (2015) 'Impact of virulence genes on sepsis severity and survival in *Escherichia coli* bacteremia', *Virulence*, 6(1), pp. 93–100. doi: 10.4161/21505594.2014.991234.

Moreno, E., Planells, I., Prats, G., Planes, A. M., Moreno, G. and Andreu, A. (2005)

‘Comparative study of Escherichia coli virulence determinants in strains causing urinary tract bacteremia versus strains causing pyelonephritis and other sources of bacteremia’, *Diagnostic Microbiology and Infectious Disease*, 53(2), pp. 93–99. doi: 10.1016/j.diagmicrobio.2005.05.015.

Morgan, S. D. (2007) *A STUDY OF THE DEVELOPMENT OF CRYSTALLINE M IR A B 1L ISBIOFILMS ON URINARY CATHETERS*. Cardiff University.

Morkaew, T., Pinyakong, O. and Tachaboonyakiat, W. (2017) ‘Structural effect of quaternary ammonium chitin derivatives on their bactericidal activity and specificity’, *International Journal of Biological Macromolecules*, 101, pp. 719–728. doi: 10.1016/j.ijbiomac.2017.03.159.

Morton, L. D., McLaughlin, G. L. and Whiteley, H. E. (1991) ‘Effects of temperature, amebic strain, and carbohydrates on Acanthamoeba adherence to corneal epithelium in vitro.’, *Infection and immunity*, 59(10), pp. 3819–22. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1894379 (Accessed: 13 December 2018).

Mshana, S. E., Imirzalioglu, C., Hossain, H., Hain, T., Domann, E. and Chakraborty, T. (2009) ‘Conjugative IncFI plasmids carrying CTX-M-15 among Escherichia coli ESBL producing isolates at a University hospital in Germany.’, *BMC infectious diseases*, 9(1), p. 97. doi: 10.1186/1471-2334-9-97.

Müller, A., Walochnik, J., Wagner, M. and Schmitz-Esser, S. (2016) ‘A clinical Acanthamoeba isolate harboring two distinct bacterial endosymbionts’, *European Journal of Protistology*, 56, pp. 21–25. doi: 10.1016/j.ejop.2016.04.002.

Mulvey, M. a. (2002) ‘Adhesion and entry of uropathogenic Escherichia coli’, *Cellular Microbiology*, 4(5), pp. 257–271. doi: 10.1046/j.1462-5822.2002.00193.x.

Mulvey, M. A., Schilling, J. D. and Hultgren, S. J. (2001) 'Establishment of a Persistent Escherichia coli Reservoir during the Acute Phase of a Bladder Infection', *Infection and Immunity*, 69(7), pp. 4572–4579. doi: 10.1128/IAI.69.7.4572-4579.2001.

Naas, T., Poirel, L. and Nordmann, P. (2008) 'Minor extended-spectrum beta-lactamases.', *Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases*, 14 Suppl 1, pp. 42–52. doi: 10.1111/j.1469-0691.2007.01861.x.

Naber, K. G., Schito, G., Botto, H., Palou, J. and Mazzei, T. (2008) 'Surveillance study in Europe and Brazil on clinical aspects and Antimicrobial Resistance Epidemiology in Females with Cystitis (ARESC): implications for empiric therapy.', *European urology*, 54(5), pp. 1164–75. doi: 10.1016/j.eururo.2008.05.010.

Nagamatsu, K., Hannan, T. J., Guest, R. L., Kostakioti, M., Hadjifrangiskou, M., Binkley, J., Dodson, K., Raivio, T. L. and Hultgren, S. J. (2015) 'Dysregulation of *Escherichia coli* α-hemolysin expression alters the course of acute and persistent urinary tract infection', *Proceedings of the National Academy of Sciences*, 112(8), pp. E871–E880. doi: 10.1073/pnas.1500374112.

Nagano, K. and Nikaido, H. (2009) 'Kinetic behavior of the major multidrug efflux pump AcrB of Escherichia coli', *Proceedings of the National Academy of Sciences*, 106(14), pp. 5854–5858. doi: 10.1073/pnas.0901695106.

Narciso, A., Nunes, F., Amores, T., Lito, L., Melo-Cristino, J. and Duarte, A. (2012) 'Persistence of uropathogenic Escherichia coli strains in the host for long periods of time: relationship between phylogenetic groups and virulence factors', *European Journal of Clinical Microbiology & Infectious Diseases*, 31(6), pp. 1211–1217. doi: 10.1007/s10096-011-1431-7.

NASEER, U., HALDORSEN, B., TOFTELAND, S., HEGSTAD, K., SCHEUTZ, F., SIMONSEN, G. S., SUNDSFJORD, A. and Norwegian ESBL Study Group (2009) 'Molecular characterization of CTX-M-15-producing clinical isolates of Escherichia coli reveals the spread of multidrug-resistant ST131 (O25:H4) and ST964 (O102:H6) strains in Norway', *APMIS*, 117(7), pp. 526–536. doi: 10.1111/j.1600-0463.2009.02465.x.

Neff, R. J. and Neff, R. H. (1969) 'The biochemistry of amoebic encystment.', *Symposia of the Society for Experimental Biology*, 23, pp. 51–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/4243920 (Accessed: 28 May 2015).

Negroni, A., Cucchiara, S. and Stronati, L. (2015) 'Apoptosis, Necrosis, and Necroptosis in the Gut and Intestinal Homeostasis', *Mediators of Inflammation*. Hindawi, 2015, pp. 1–10. doi: 10.1155/2015/250762.

Nekrasova, O., Yakimov, S., Kirpichnikov, M. and Feofanov, A. (2018) 'Recombinant scorpion toxins: Focus on four-disulfide peptide blockers of Kv1-channels', *Bioengineered*, 9(1), pp. 25–29. doi: 10.1080/21655979.2017.1373530.

Nero, L. C., Tarver, M. G. and Hedrick, L. R. (1964) 'Growth of Acanthamoeba Castellani With the Yeast Torulopsis Famata.', *Journal of bacteriology*, 87(1), pp. 220–225.

Newire, E. A., Ahmed, S. F., House, B., Valiente, E. and Pimentel, G. (2013) 'Detection of new SHV-12, SHV-5 and SHV-2a variants of extended spectrum Beta-lactamase in Klebsiella pneumoniae in Egypt', *Annals of Clinical Microbiology and Antimicrobials*, 12(1), p. 16. doi: 10.1186/1476-0711-12-16.

Nicolle, L. E. (2001) 'Urinary tract infections in long-term-care facilities.', *Infection control and hospital epidemiology*, 22(3), pp. 167–75. doi: 10.1086/501886.

Nicolle, L. E. (2014) 'Catheter associated urinary tract infections.', *Antimicrobial resistance and*

infection control. BioMed Central Ltd, 3(1), p. 23. doi: 10.1186/2047-2994-3-23.

Nipič, D., Podlesek, Z., Budič, M., črnigoj, M. and Žgur-Bertok, D. (2013) 'Escherichia coli Uropathogenic-Specific Protein, Usp, Is a Bacteriocin-Like Genotoxin', *The Journal of Infectious Diseases*, 208(10), pp. 1545–1552. doi: 10.1093/infdis/jit480.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Mohebali, M., Maghsood, A. H., Motevalli-Haghi, A., Martín-Navarro, C. M., Farnia, S., Valladares, B. and Rezaeian, M. (2009) 'Genotyping of Acanthamoeba isolates from clinical and environmental specimens in Iran', *Experimental Parasitology*, 121(3), pp. 242–245. doi: 10.1016/j.exppara.2008.11.003.

Niyyati, M., Saberi, R., Latifi, A. and Lasjerdi, Z. (2016) 'Distribution of Acanthamoeba Genotypes Isolated from Recreational and Therapeutic Geothermal Water Sources in Southwestern Iran', *Environmental Health Insights*, 10, p. EHI.S38349. doi: 10.4137/EHI.S38349.

Norinder, B. S., Köves, B., Yadav, M., Brauner, A. and Svanborg, C. (2012) 'Do Escherichia coli strains causing acute cystitis have a distinct virulence repertoire?', *Microbial Pathogenesis*, 52(1), pp. 10–16. doi: 10.1016/j.micpath.2011.08.005.

Nowicki, B., Labigne, A., Moseley, S., Hull, R., Hull, S. and Moulds, J. (1990) 'The Dr hemagglutinin, afimbrial adhesins AFA-I and AFA-III, and F1845 fimbriae of uropathogenic and diarrhea-associated Escherichia coli belong to a family of hemagglutinins with Dr receptor recognition.', *Infection and immunity*, 58(1), pp. 279–81. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1967170 (Accessed: 23 December 2018).

Nuprasert, W., Putaporntip, C., Pariyakanok, L. and Jongwutiwes, S. (2010) 'Identification of a

novel t17 genotype of acanthamoeba from environmental isolates and t10 genotype causing keratitis in Thailand.', *Journal of clinical microbiology*, 48(12), pp. 4636–40. doi: 10.1128/JCM.01090-10.

Nyberg, S. D., Österblad, M., Hakanen, A. J., Huovinen, P., Jalava, J. and THE FINNISH STUDY GROUP FOR ANTIMIC (2007) 'Detection and molecular genetics of extended-spectrum beta-lactamases among cefuroxime-resistant Escherichia coli and Klebsiella spp. isolates from Finland, 2002–2004', *Scandinavian Journal of Infectious Diseases*, 39(5), pp. 417–424. doi: 10.1080/00365540601105731.

O'Farrell, P. H. (1975) 'High resolution two-dimensional electrophoresis of proteins.', *The Journal of biological chemistry*, 250(10), pp. 4007–21. Available at: http://www.ncbi.nlm.nih.gov/pubmed/236308 (Accessed: 30 December 2018).

Olesen, B., Scheutz, F., Andersen, R. L., Menard, M., Boisen, N., Johnston, B., Hansen, D. S., Krogfelt, K. A., Nataro, J. P. and Johnson, J. R. (2012) 'Enteroaggregative Escherichia coli O78:H10, the Cause of an Outbreak of Urinary Tract Infection', *Journal of Clinical Microbiology*, 50(11), pp. 3703–3711. doi: 10.1128/JCM.01909-12.

Omaña-Molina, M., González-Robles, A., Iliana Salazar-Villatoro, L., Lorenzo-Morales, J., Cristóbal-Ramos, A. R., Hernández-Ramírez, V. I., Talamás-Rohana, P., Méndez Cruz, A. R. and Martínez-Palomo, A. (2013) 'Reevaluating the role of Acanthamoeba proteases in tissue invasion: observation of cytopathogenic mechanisms on MDCK cell monolayers and hamster corneal cells.', *BioMed research international*. Hindawi Limited, 2013, p. 461329. doi: 10.1155/2013/461329.

Omaña-Molina, M., Hernandez-Martinez, D., Sanchez-Rocha, R., Cardenas-Lemus, U., Salinas-Lara, C., Mendez-Cruz, A. R., Colin-Barenque, L., Aley-Medina, P., Espinosa-Villanueva, J., Moreno-Fierros, L. and Lorenzo-Morales, J. (2017) 'In vivo CNS infection model of

Acanthamoeba genotype T4: the early stages of infection lack presence of host inflammatory response and are a slow and contact-dependent process', *Parasitology Research*, 116(2), pp. 725–733. doi: 10.1007/s00436-016-5338-1.

Omaña-Molina, M., Vanzzini-Zago, V., Hernández-Martínez, D., Reyes-Batlle, M., Castelan-Ramírez, I., Hernández-Olmos, P., Salazar-Villatoro, L., González-Robles, A., Ramírez-Flores, E., Servín-Flores, C., Flores-Alvarado, V., Alcántara-Castro, M. and Lorenzo-Morales, J. (2018) 'Acanthamoeba keratitis in Mexico: Report of a clinical case and importance of sensitivity assays for a better outcome', *Experimental Parasitology*, 196, pp. 22–27. doi: 10.1016/j.exppara.2018.11.005.

Orskov, F. and Orskov, I. (1992) 'Escherichia coli serotyping and disease in man and animals.', *Canadian journal of microbiology*, 38(7), pp. 699–704. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1382824 (Accessed: 1 January 2019).

Orskov, I., Orskov, F., Jann, B. and Jann, K. (1977) 'Serology, chemistry, and genetics of O and K antigens of Escherichia coli.', *Bacteriological reviews*, 41(3), pp. 667–710. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=414020&tool=pmcentrez&rendertype=abstract (Accessed: 20 April 2015).

Oueslati, S., Nordmann, P. and Poirel, L. (2015) 'Heterogeneous hydrolytic features for OXA-48-like -lactamases', *Journal of Antimicrobial Chemotherapy*, 163(January), pp. 1059–1063. doi: 10.1093/jac/dku524.

Ovchinnikova, O., Doyle, L., Huang, B.-S., Kimber, M., Lowary, T. and Whitfield, C. (2016) 'Biochemical Characterization of Bifunctional 3-Deoxy-β-d-manno-oct-2-ulosonic Acid (β-Kdo) Transferase KpsC from Escherichia coli Involved in Capsule Biosynthesis.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 291(41), pp. 21519–21530. doi: 10.1074/jbc.M116.751115.

Ozkoc, S., Tuncay, S., Delibas, S. B., Akisu, C., Ozbek, Z., Durak, I. and Walochnik, J. (2008) 'Identification of Acanthamoeba genotype T4 and Paravahlkampfia sp. from two clinical samples', *Journal of Medical Microbiology*, 57(3), pp. 392–396. doi: 10.1099/jmm.0.47650-0.

Özpinar, N., Özcelik, S. and Yunlu, Ö. (2017) 'Isolation and morphotyping of Acanthamoeba spp. and Vermamoeba spp. from hospital air-conditioning systems', *Cumhuriyet Medical Journal*, 39(1), pp. 369–369. doi: 10.7197/cmj.v39i1.5000194070.

Padzik, M., Baltaza, W., Szaflik, J. P., Hendiger, E., Dybicz, M. and Chomicz, L. (2018) 'Comparison of chlorhexidine disinfectant in vitro effect on environmental and ocular Acanthamoeba strains, the amoebic agents of human keratitis − an emerging sight-threatening corneal disease in Poland', *Annals of parasitology*, 64(3), pp. 229–233. doi: 10.17420/ap6403.157.

Pallett, A. and Hand, K. (2010) 'Complicated urinary tract infections: Practical solutions for the treatment of multiresistant gram-negative bacteria', *Journal of Antimicrobial Chemotherapy*, 65(SUPPL. 3). doi: 10.1093/jac/dkq298.

Palmer, J., Flint, S. and Brooks, J. (2007) 'Bacterial cell attachment, the beginning of a biofilm', *Journal of Industrial Microbiology & Biotechnology*, 34(9), pp. 577–588. doi: 10.1007/s10295-007-0234-4.

Panjwani, N. (2010) 'Pathogenesis of acanthamoeba keratitis.', *The ocular surface*. NIH Public Access, 8(2), pp. 70–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/20427010 (Accessed: 13 December 2018).

Paquet, V. E. and Charette, S. J. (2016) 'Amoeba-resisting bacteria found in multilamellar bodies secreted by Dictyostelium discoideum: Social amoebae can also package bacteria', *FEMS*

Microbiology Ecology. Edited by R. Kümmerli. Oxford University Press, 92(3), p. fiw025. doi: 10.1093/femsec/fiw025.

Parent, M. E. and Velegol, D. (2004) 'E. coli adhesion to silica in the presence of humic acid', *Colloids and Surfaces B: Biointerfaces*, 39(1–2), pp. 45–51. doi: 10.1016/j.colsurfb.2004.08.020.

Park, Y. S., Bae, I. K., Kim, J., Jeong, S. H., Hwang, S. S., Seo, Y. H., Cho, Y. K., Lee, K. and Kim, J. M. (2014) 'Risk factors and molecular epidemiology of community-onset extended-spectrum β-lactamase-producing Escherichia coli Bacteremia', *Yonsei Medical Journal*, 55(2), pp. 467–475. doi: 10.3349/ymj.2014.55.2.467.

Paterson, D. L. and Bonomo, R. A. (2005) 'Extended-spectrum beta-lactamases: a clinical update.', *Clinical microbiology reviews*, 18(4), pp. 657–686. doi: 10.1128/CMR.18.4.657-686.2005.

Pereira, D. A., Vidotto, M. C., Nascimento, K. A., Santos, A. C. R. dos, Mechler, M. L., Oliveira, L. G. de, Pereira, D. A., Vidotto, M. C., Nascimento, K. A., Santos, A. C. R. dos, Mechler, M. L. and Oliveira, L. G. de (2016) 'Virulence factors of Escherichia coli in relation to the importance of vaccination in pigs', *Ciência Rural*. Universidade Federal de Santa Maria, 46(8), pp. 1430–1437. doi: 10.1590/0103-8478cr20151269.

Philippon, A., Arlet, G. and Jacoby, G. A. (2002) 'Plasmid-Determined AmpC-Type -Lactamases', *Antimicrobial Agents and Chemotherapy*, 46(1), pp. 1–11. doi: 10.1128/AAC.46.1.1-11.2002.

Pichon, C., Héchard, C., du Merle, L., Chaudray, C., Bonne, I., Guadagnini, S., Vandewalle, A. and Le Bouguénec, C. (2009) 'Uropathogenic Escherichia coli AL511 requires flagellum to enter renal collecting duct cells.', *Cellular microbiology*, 11(4), pp. 616–28. doi:

10.1111/j.1462-5822.2008.01278.x.

Pitout, J. D. D. (2012) ‘Extraintestinal Pathogenic Escherichia coli: A Combination of Virulence with Antibiotic Resistance.’, *Frontiers in microbiology*. Frontiers, 3, p. 9. doi: 10.3389/fmicb.2012.00009.

Pitout, J. D. D., Laupland, K. B., Church, D. L., Menard, M. L. and Johnson, J. R. (2005) ‘Virulence Factors of Escherichia coli Isolates That Produce CTX-M-Type Extended-Spectrum -Lactamases’, *Antimicrobial Agents and Chemotherapy*, 49(11), pp. 4667–4670. doi: 10.1128/AAC.49.11.4667-4670.2005.

Plowman, R., Graves, N., Griffin, M. A., Roberts, J. A., Swan, A. V, Cookson, B. and Taylor, L. (2001) ‘The rate and cost of hospital-acquired infections occurring in patients admitted to selected specialties of a district general hospital in England and the national burden imposed.’, *The Journal of hospital infection*, 47(3), pp. 198–209. doi: 10.1053/jhin.2000.0881.

Poirel, L., Potron, A. and Nordmann, P. (2012) ‘OXA-48-like carbapenemases: The phantom menace’, *Journal of Antimicrobial Chemotherapy*, 67(7), pp. 1597–1606. doi: 10.1093/jac/dks121.

Poon, I. K. H., Hulett, M. D. and Parish, C. R. (2010) ‘Molecular mechanisms of late apoptotic/necrotic cell clearance’, *Cell Death & Differentiation*, 17(3), pp. 381–397. doi: 10.1038/cdd.2009.195.

Proca-Ciobanu, M., Lupascu, G., Petrovici, A. and Ionescu, M. D. (1975) ‘Electron microscopic study of a pathogenic Acanthamoeba castellani strain: The presence of bacterial endosymbionts’, *International Journal for Parasitology*. Pergamon, 5(1), pp. 49–56. doi: 10.1016/0020-7519(75)90097-1.

Public Health England (2016a) *Carbapenemase-producing Enterobacteriaceae: laboratory confirmed cases, 2003 to 2015 - GOV.UK*. Available at: https://www.gov.uk/government/publications/carbapenemase-producing-enterobacteriaceae-laboratory-confirmed-cases/carbapenemase-producing-enterobacteriaceae-laboratory-confirmed-cases-2003-to-2013 (Accessed: 10 December 2018).

Public Health England (2016b) *UK standards for microbiology investigations*. Available at: https://www.gov.uk/government/groups/standards-for-microbiology-investigations- (Accessed: 14 March 2019).

Pulss, S., Stolle, I., Stamm, I., Leidner, U., Heydel, C., Semmler, T., Prenger-Berninghoff, E. and Ewers, C. (2018) 'Multispecies and Clonal Dissemination of OXA-48 Carbapenemase in Enterobacteriaceae From Companion Animals in Germany, 2009-2016.', *Frontiers in microbiology*. Frontiers Media SA, 9, p. 1265. doi: 10.3389/fmicb.2018.01265.

Raeispour, M. and Ranjbar, R. (2018) 'Antibiotic resistance, virulence factors and genotyping of Uropathogenic Escherichia coli strains.', *Antimicrobial resistance and infection control*. BioMed Central, 7, p. 118. doi: 10.1186/s13756-018-0411-4.

Raetz, C. R. H. and Whitfield, C. (2002) 'Lipopolysaccharide endotoxins.', *Annual review of biochemistry*, 71, pp. 635–700. doi: 10.1146/annurev.biochem.71.110601.135414.

Rahman, M. M., Yagita, K., Kobayashi, A., Oikawa, Y., Hussein, A. I. A., Matsumura, T. and Tokoro, M. (2013) 'Genetic Characterization of Clinical Acanthamoeba Isolates from Japan using Nuclear and Mitochondrial Small Subunit Ribosomal RNA', *The Korean Journal of Parasitology*, 51(4), pp. 401–411. doi: 10.3347/kjp.2013.51.4.401.

Ramadan, D. S., Bassyoni, E. A., Amer, M. M. and Emam, S. M. (2016) 'Detection of ESBL

Producing Bacteria in Cases of Urinary Tract Infection in Pediatric Department at Benha University Hospital', *Egyptian Journal of Medical Microbiology*, 25(1), pp. 77–84.

Ransom, J. R., Belk, K. E., Sofos, J. N., Stopforth, J. D., Scanga, J. A. and Smith, G. C. (2003) 'Comparison of Intervention Technologies for Reducing Escherichia coli 0 157:H7 on Beef Cuts and Trimmings', *Food protection trends*. International Association for Food Protection, (23), p. 24–34. Available at: http://agris.fao.org/agris-search/search.do?recordID=US201400175030 (Accessed: 1 January 2019).

Rathmell, J. C. and Thompson, C. B. (2002) 'Pathways of apoptosis in lymphocyte development, homeostasis, and disease.', *Cell*, 109 Suppl, pp. S97-107. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11983156 (Accessed: 12 December 2018).

Rawat, D. and Nair, D. (2010a) 'Extended-spectrum ß-lactamases in gram negative bacteria', *Journal of Global Infectious Diseases*, 2(3), p. 263. doi: 10.4103/0974-777X.68531.

Rawat, D. and Nair, D. (2010b) 'Extended-spectrum β-lactamases in Gram Negative Bacteria.', *Journal of global infectious diseases*. Wolters Kluwer -- Medknow Publications, 2(3), pp. 263–74. doi: 10.4103/0974-777X.68531.

Reed, P. W., Densmore, A. and Bloch, R. J. (2012) 'Optimization of large gel 2D electrophoresis for proteomic studies of skeletal muscle.', *Electrophoresis*. NIH Public Access, 33(8), pp. 1263–70. doi: 10.1002/elps.201100642.

Reinert, R. R., Low, D. E., Rossi, F., Zhang, X., Wattal, C. and Dowzicky, M. J. (2007) 'Antimicrobial susceptibility among organisms from the Asia/Pacific Rim, Europe and Latin and North America collected as part of TEST and the in vitro activity of tigecycline', *Journal of Antimicrobial Chemotherapy*, 60(5), pp. 1018–1029. doi: 10.1093/jac/dkm310.

Reppin, F., Cochet, S., El Nemer, W., Fritz, G. and Schmidt, G. (2017) 'High Affinity Binding

of Escherichia coli Cytotoxic Necrotizing Factor 1 (CNF1) to Lu/BCAM Adhesion Glycoprotein', *Toxins*, 10(1), p. 3. doi: 10.3390/toxins10010003.

Retana-Moreira, L., Abrahams-Sandí, E., Castro-Artavia, E., Fernández-Sánchez, A., Castro-Castillo, A., Reyes-Batlle, M. and Lorenzo-Morales, J. (2015) 'Isolation and Molecular Characterization of Acanthamoeba Strains from Dental Units in Costa Rica', *Journal of Eukaryotic Microbiology*, 62(6), pp. 733–736. doi: 10.1111/jeu.12229.

Reyes-Batlle, M., Todd, C. D., Martín-Navarro, C. M., López-Arencibia, A., Cabello-Vilchez, A. M., González, A. C., Córdoba-Lanús, E., Lindo, J. F., Valladares, B., Piñero, J. E. and Lorenzo-Morales, J. (2014) 'Isolation and characterization of Acanthamoeba strains from soil samples in Gran Canaria, Canary Islands, Spain', *Parasitology Research*, 113(4), pp. 1383–1388. doi: 10.1007/s00436-014-3778-z.

Rice, J. C., Peng, T., Kuo, Y.-F., Pendyala, S., Simmons, L., Boughton, J., Ishihara, K., Nowicki, S. and Nowicki, B. J. (2006) 'Renal allograft injury is associated with urinary tract infection caused by Escherichia coli bearing adherence factors.', *American journal of transplantation : official journal of the American Society of Transplantation and the American Society of Transplant Surgeons*, 6(10), pp. 2375–83. doi: 10.1111/j.1600-6143.2006.01471.x.

Rice, L. B. (2012) 'Mechanisms of resistance and clinical relevance of resistance to β-lactams, glycopeptides, and fluoroquinolones.', *Mayo Clinic proceedings*, 87(2), pp. 198–208. doi: 10.1016/j.mayocp.2011.12.003.

Rittig, M. G., Kaufmann, A., Robins, A., Shaw, B., Sprenger, H., Gemsa, D., Foulongne, V., Rouot, B. and Dornand, J. (2003) 'Smooth and rough lipopolysaccharide phenotypes of Brucella induce different intracellular trafficking and cytokine/chemokine release in human monocytes.', *Journal of leukocyte biology*, 74(6), pp. 1045–55. doi: 10.1189/jlb.0103015.

Robino, L., Scavone, P., Araujo, L., Algorta, G., Zunino, P. and Vignoli, R. (2013) 'Detection of intracellular bacterial communities in a child with escherichia coli recurrent urinary tract infections', *Pathogens and Disease*. Oxford University Press, 68(3), pp. 78–81. doi: 10.1111/2049-632X.12047.

Rocha-Cabrera, P., Reyes-Batlle, M., Martín-Navarro, C. M., Dorta-Gorrín, A., López-Arencibia, A., Sifaoui, I., Martínez-Carretero, E., Piñero, J. E., Martín-Barrera, F., Valladares, B. and Lorenzo-Morales, J. (2015) 'Detection of Acanthamoeba on the ocular surface in a Spanish population using the Schirmer strip test: Pathogenic potential, molecular classification and evaluation of the sensitivity to chlorhexidine and voriconazole of the isolated Acanthamoeba strains', *Journal of Medical Microbiology*. doi: 10.1099/jmm.0.000103.

Rodríguez-Pérez, E. G., Escandón-Vargas, K. and Ancer, A. (2017) 'Granulomatous amebic encephalitis caused by Acanthamoeba sp. in an immunocompetent Mexican adult', *Revista da Sociedade Brasileira de Medicina Tropical*, 50(3), pp. 432–432. doi: 10.1590/0037-8682-0166-2016.

Rodriguez-Siek, K. E., Giddings, C. W., Doetkott, C., Johnson, T. J., Fakhr, M. K. and Nolan, L. K. (2005) 'Comparison of Escherichia coli isolates implicated in human urinary tract infection and avian colibacillosis', *Microbiology*, 151(6), pp. 2097–2110. doi: 10.1099/mic.0.27499-0.

Rodríguez-Zaragoza, S. (1994) 'Ecology of Free-Living Amoebae', *Critical Reviews in Microbiology*, 20(3), pp. 225–241. doi: 10.3109/10408419409114556.

Rosen, D. A., Hooton, T. M., Stamm, W. E., Humphrey, P. A. and Hultgren, S. J. (2007) 'Detection of Intracellular Bacterial Communities in Human Urinary Tract Infection', *PLoS Medicine*. Edited by S. M. Opal. Public Library of Science, 4(12), p. e329. doi: 10.1371/journal.pmed.0040329.

Rosenberg, K., Bertaux, J., Krome, K., Hartmann, A., Scheu, S. and Bonkowski, M. (2009) 'Soil amoebae rapidly change bacterial community composition in the rhizosphere of Arabidopsis thaliana', *The ISME Journal*, 3(6), pp. 675–684. doi: 10.1038/ismej.2009.11.

Ruppé, E., Pitsch, A., Tubach, F., Lastours, V., Chau, F., Pasquet, B., Lucet, J.-C., Andremont, A. and Fantin, B. (2012) 'Clinical predictive values of extended-spectrum beta-lactamase carriage in patients admitted to medical wards', *European Journal of Clinical Microbiology & Infectious Diseases*, 31(3), pp. 319–325. doi: 10.1007/s10096-011-1313-z.

Russo, T. A. and Johnson, J. R. (2000) 'Proposal for a New Inclusive Designation for Extraintestinal Pathogenic Isolates of *Escherichia coli:* ExPEC', *The Journal of Infectious Diseases*, 181(5), pp. 1753–1754. doi: 10.1086/315418.

Sahuquillo-Arce, J. M., Selva, M., Perpiñán, H., Gobernado, M., Armero, C., López-Quílez, A., González, F. and Vanaclocha, H. (2011) 'Antimicrobial Resistance in More than 100,000 *Escherichia coli* Isolates According to Culture Site and Patient Age, Gender, and Location', *Antimicrobial Agents and Chemotherapy*, 55(3), pp. 1222–1228. doi: 10.1128/AAC.00765-10.

Salverda, M. L. M., De Visser, J. A. G. M. and Barlow, M. (2010) 'Natural evolution of TEM-1 β-lactamase: experimental reconstruction and clinical relevance', *FEMS Microbiology Reviews*, 34(6), pp. 1015–1036. doi: 10.1111/j.1574-6976.2010.00222.x.

Samadpour, M. (1995) 'Molecular epidemiology of Escherichia coli O157:H7 by restriction fragment length polymorphism using Shiga-like toxin genes.', *Journal of clinical microbiology*, 33(8), pp. 2150–4. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=228353&tool=pmcentrez&rendertype=abstract (Accessed: 25 May 2015).

Sandström, G., Saeed, A. and Abd, H. (2011) ‘Acanthamoeba-bacteria: a model to study host interaction with human pathogens.’, *Current drug targets*, 12(7), pp. 936–41. Available at: http://www.ncbi.nlm.nih.gov/pubmed/21366523 (Accessed: 30 December 2018).

Sandt, C., Barbeau, J., Gagnon, M.-A. and Lafleur, M. (2007) ‘Role of the ammonium group in the diffusion of quaternary ammonium compounds in Streptococcus mutans biofilms’, *Journal of Antimicrobial Chemotherapy*, 60(6), pp. 1281–1287. doi: 10.1093/jac/dkm382.

Santos, L. C., Oliveira, M. S., Lobo, R. D., Higashino, H. R., Costa, S. F., Van Der Heijden, I. M., Giudice, M. C., Silva, A. R. and Levin, A. S. (2009) ‘Acanthamoeba spp. in urine of critically ill patients’, *Emerging Infectious Diseases*, pp. 1144–1146. doi: 10.3201/eid1507.081415.

Sauvage, E. and Terrak, M. (2016) ‘Glycosyltransferases and Transpeptidases/Penicillin-Binding Proteins: Valuable Targets for New Antibacterials’, *Antibiotics*, 5(1), p. 12. doi: 10.3390/antibiotics5010012.

Savas, S., Kucukyılmaz, E., U Celik, E. and Ates, M. (2015) ‘Effects of different antibacterial agents on enamel in a biofilm caries model.’, *Journal of oral science*, 57(4), pp. 367–72. doi: 10.2334/josnusd.57.367.

Schmidtke, A. J. and Hanson, N. D. (2006) ‘Model system to evaluate the effect of ampD mutations on AmpC-mediated beta-lactam resistance.’, *Antimicrobial agents and chemotherapy*, 50(6), pp. 2030–7. doi: 10.1128/AAC.01458-05.

Schmiedel, J., Falgenhauer, L., Domann, E., Bauerfeind, R., Prenger-Berninghoff, E., Imirzalioglu, C. and Chakraborty, T. (2014) ‘Multiresistant extended-spectrum β-lactamase-producing Enterobacteriaceae from humans, companion animals and horses in central Hesse, Germany’, *BMC Microbiology*, 14(1), p. 187. doi: 10.1186/1471-2180-14-187.

Scholes, D., Hooton, T. M., Roberts, P. L., Stapleton, A. E., Gupta, K. and Stamm, W. E. (2000) 'Risk factors for recurrent urinary tract infection in young women.', *The Journal of infectious diseases*, 182(4), pp. 1177–82. doi: 10.1086/315827.

Schroeder, J. M., Booton, G. C., Hay, J., Niszl, I. A., Seal, D. V., Markus, M. B., Fuerst, P. A. and Byers, T. J. (2001) 'Use of Subgenic 18S Ribosomal DNA PCR and Sequencing for Genus and Genotype Identification of Acanthamoebae from Humans with Keratitis and from Sewage Sludge', *Journal of Clinical Microbiology*, 39(5), pp. 1903–1911. doi: 10.1128/JCM.39.5.1903-1911.2001.

Schwan, W. R. (2008) 'Flagella allow uropathogenic Escherichia coli ascension into murine kidneys.', *International journal of medical microbiology : IJMM*, 298(5–6), pp. 441–7. doi: 10.1016/j.ijmm.2007.05.009.

Sciences, C. (2008) 'Acanthamoeba pathogenesis', (April).

Scott, V. C. S., Haake, D. A., Churchill, B. M., Justice, S. S. and Kim, J.-H. (2015) 'Intracellular Bacterial Communities: A Potential Etiology for Chronic Lower Urinary Tract Symptoms', *Urology*, 86(3), pp. 425–431. doi: 10.1016/j.urology.2015.04.002.

Sekiguchi, J. and Yamamoto, H. (2012) *The Frontiers of Molecular Microbiology Revisited*.

Sente, C., Erume, J., Naigaga, I., Magambo, P. K., Ochwo, S., Mulindwa, J., Namara, B. G., Kato, C. D., Sebyatika, G., Muwonge, K. and Ocaido, M. (2016) 'Occurrence and genetic characterisation of Acanthamoeba spp. from environmental and domestic water sources in Queen Elizabeth Protected Area, Uganda.', *Parasites & vectors*. BioMed Central, 9, p. 127. doi: 10.1186/s13071-016-1411-y.

Serra, R., Grande, R., Butrico, L., Rossi, A., Settimio, U. F., Caroleo, B., Amato, B., Gallelli, L. and de Franciscis, S. (2015) 'Chronic wound infections: the role of *Pseudomonas*

aeruginosa and *Staphylococcus aureus*', *Expert Review of Anti-infective Therapy*, 13(5), pp. 605–613. doi: 10.1586/14787210.2015.1023291.

Servin, A. L. (2005) 'Pathogenesis of Afa/Dr diffusely adhering Escherichia coli.', *Clinical microbiology reviews*. American Society for Microbiology (ASM), 18(2), pp. 264–92. doi: 10.1128/CMR.18.2.264-292.2005.

Servin, A. L. (2014) 'Pathogenesis of Human Diffusely Adhering Escherichia coli Expressing Afa/Dr Adhesins (Afa/Dr DAEC): Current Insights and Future Challenges', *Clinical Microbiology Reviews*, 27(4), pp. 823–869. doi: 10.1128/CMR.00036-14.

Shapiro, A. B. (2017) 'Kinetics of Sulbactam Hydrolysis by β-Lactamases, and Kinetics of β-Lactamase Inhibition by Sulbactam.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 61(12). doi: 10.1128/AAC.01612-17.

Sheng, W.-H., Hung, C.-C., Huang, H.-H., Liang, S.-Y., Cheng, Y.-J., Ji, D.-D. and Chang, S.-C. (2009) 'First case of granulomatous amebic encephalitis caused by Acanthamoeba castellanii in Taiwan.', *The American journal of tropical medicine and hygiene*, 81(2), pp. 277–9. Available at: http://www.ncbi.nlm.nih.gov/pubmed/19635883 (Accessed: 24 December 2018).

Shibl, A., Al-Agamy, M., Memish, Z., Senok, A., Khader, S. A. and Assiri, A. (2013) 'The emergence of OXA-48- and NDM-1-positive Klebsiella pneumoniae in Riyadh, Saudi Arabia', *International Journal of Infectious Diseases*, 17(12), pp. e1130–e1133. doi: 10.1016/j.ijid.2013.06.016.

Shirai, A., Maeda, T., Itoh, M., Kawano, G. and Kourai, H. (2000) 'Control of Legionella Species and Host Amoeba by Bis-quaternary Ammonium Compounds', *Biocontrol Science*, 5(2), pp. 94–102. Available at: https://www.jstage.jst.go.jp/article/bio1996/5/2/5_2_97/_pdf

(Accessed: 12 March 2019).

Shrestha, S., Tada, T., Miyoshi-Akiyama, T., Ohara, H., Shimada, K., Satou, K., Teruya, K., Nakano, K., Shiroma, A., Sherchand, J. B., Rijal, B. P., Hirano, T., Kirikae, T. and Pokhrel, B. M. (2015) 'Molecular epidemiology of multidrug-resistant Acinetobacter baumannii isolates in a university hospital in Nepal reveals the emergence of a novel epidemic clonal lineage', *International Journal of Antimicrobial Agents*, 46(5), pp. 526–531. doi: 10.1016/j.ijantimicag.2015.07.012.

Siddiqui, R., Aqeel, Y. and Khan, N. A. (2016) 'The Development of Drugs against Acanthamoeba Infections.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology Journals, 60(11), pp. 6441–6450. doi: 10.1128/AAC.00686-16.

Siddiqui, R. and Khan, N. (2012) *Biology and pathogenesis of Acanthamoeba*, *Parasites & Vectors*. BioMed Central Ltd. doi: 10.1186/1756-3305-5-6.

Sign (2006) 'Management of suspected bacterial urinary tract infection in adults. A national clinical guideline', (July). Available at: www.sign.ac.uk/pdf/sign88.pdf.

Silvaggi, N. R., Kaur, K., Adediran, S. A., Pratt, R. F. and Kelly, J. A. (2004) 'Toward better antibiotics: Crystallographic studies of a novel class of DD-peptidase/β-lactamase inhibitors', *Biochemistry*, 43(22), pp. 7046–7053. doi: 10.1021/bi049612c.

Sissons, J., Alsam, S., Goldsworthy, G., Lightfoot, M., Jarroll, E. L. and Khan, N. A. (2006) 'Identification and properties of proteases from an Acanthamoeba isolate capable of producing granulomatous encephalitis.', *BMC microbiology*, 6, p. 42. doi: 10.1186/1471-2180-6-42.

Sissons, J., Kim, K. S., Stins, M., Alsam, S., Khan, N. A. and Jayasekera, S. (2005) 'Acanthamoeba castellanii Induces Host Cell Death via a Phosphatidylinositol 3-Kinase-

Dependent Mechanism Acanthamoeba castellanii Induces Host Cell Death via a Phosphatidylinositol 3-Kinase-Dependent Mechanism', 73(5), pp. 1–6. doi: 10.1128/IAI.73.5.2704.

Skjøt-Rasmussen, L., Olsen, S. S., Jakobsen, L., Ejrnæs, K., Scheutz, F., Lundgren, B., Frimodt-Møller, N. and Hammerum, A. M. (2013) 'Escherichia coli clonal group A causing bacteraemia of urinary tract origin', *Clinical Microbiology and Infection*, 19(7), pp. 656–661. doi: 10.1111/j.1469-0691.2012.03961.x.

Smith, C. A., Antunes, N. T., Toth, M. and Vakulenko, S. B. (2014) 'Crystal structure of carbapenemase OXA-58 from Acinetobacter baumannii.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology (ASM), 58(4), pp. 2135–43. doi: 10.1128/AAC.01983-13.

Smith, J. L., Fratamico, P. M. and Gunther, N. W. (2007) 'Extraintestinal Pathogenic *Escherichia coli*', *Foodborne Pathogens and Disease*, 4(2), pp. 134–163. doi: 10.1089/fpd.2007.0087.

Smith, M. A., Weingarten, R. A., Russo, L. M., Ventura, C. L. and O'Brien, A. D. (2015) 'Antibodies against hemolysin and cytotoxic necrotizing factor type 1 (CNF1) reduce bladder inflammation in a mouse model of urinary tract infection with toxigenic uropathogenic Escherichia coli.', *Infection and immunity*, 83(4), pp. 1661–73. doi: 10.1128/IAI.02848-14.

Smith, Y. C., Grande, K. K., Rasmussen, S. B., Brien, A. D. O. and O'Brien, A. D. (2006) 'Novel Three-Dimensional Organoid Model for Evaluation of the Interaction of Uropathogenic Escherichia coli with Terminally Differentiated Human Urothelial Cells Novel Three-Dimensional Organoid Model for Evaluation of the Interaction of Uropathogenic Esch', *Infection and immunity*, 74(1), pp. 750–757. doi:

10.1128/IAI.74.1.750.

Somily, A. M., Habib, H. A., Absar, M. M., Arshad, M. Z., Manneh, K., Al Subaie, S. S., Al Hedaithy, M. A., Sayyed, S. B., Shakoor, Z. and Murray, T. S. (2014) 'ESBL-producing Escherichia coli and Klebsiella pneumoniae at a tertiary care hospital in Saudi Arabia', *The Journal of Infection in Developing Countries*, 8(09), pp. 1129–36. doi: 10.3855/jidc.4292.

Sonnevend, Á., Ghazawi, A. A., Hashmey, R., Jamal, W., Rotimi, V. O., Shibl, A. M., Al-Jardani, A., Al-Abri, S. S., Tariq, W. U. Z., Weber, S. and Pál, T. (2015) 'Characterization of Carbapenem-Resistant Enterobacteriaceae with High Rate of Autochthonous Transmission in the Arabian Peninsula', *PLOS ONE*. Edited by A. Cloeckaert, 10(6), p. e0131372. doi: 10.1371/journal.pone.0131372.

Sougakoff, W., Goussard, S., Gerbaud, G. and Courvalin, P. (1988) 'Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes.', *Reviews of infectious diseases*, 10(4), pp. 879–84. Available at: http://www.ncbi.nlm.nih.gov/pubmed/3055179 (Accessed: 11 December 2018).

de Souza Carvalho, F. R., Carrijo-Carvalho, L. C., Chudzinski-Tavassi, A. M., Foronda, A. S. and de Freitas, D. (2011) 'Serine-like proteolytic enzymes correlated with differential pathogenicity in patients with acute Acanthamoeba keratitis', *Clinical Microbiology and Infection*, 17(4), pp. 603–609. doi: 10.1111/j.1469-0691.2010.03252.x.

Spanakos, G., Tzanetou, K., Miltsakakis, D., Patsoula, E., Malamou-Lada, E. and Vakalis, N. C. (2006) 'Genotyping of pathogenic Acanthamoebae isolated from clinical samples in Greece—Report of a clinical isolate presenting T5 genotype', *Parasitology International*, 55(2), pp. 147–149. doi: 10.1016/j.parint.2005.12.001.

Steimle, A., Autenrieth, I. B. and Frick, J.-S. (2016) 'Structure and function: Lipid A

modifications in commensals and pathogens', *International Journal of Medical Microbiology*, 306(5), pp. 290–301. doi: 10.1016/j.ijmm.2016.03.001.

Stenutz, R., Weintraub, A. and Widmalm, G. (2006) 'The structures of Escherichia coli O-polysaccharide antigens.', *FEMS microbiology reviews*, 30(3), pp. 382–403. doi: 10.1111/j.1574-6976.2006.00016.x.

Stewart, P. S. and Costerton, J. W. (2001) 'Antibiotic resistance of bacteria in biofilms.', *Lancet (London, England)*, 358(9276), pp. 135–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11463434 (Accessed: 1 January 2019).

Strelkauskas, A., Strelkauskas, J. and Moszyk-Strelkauskas, D. (2010) *Microbiology: A Clinical Approach*. 1st edn. Taylor & Francis. Available at: http://www.barnesandnoble.com/w/microbiology-anthony-strelkauskas/1101322982?ean=9780815365143 (Accessed: 22 May 2015).

Strömberg, N., Marklund, B. I., Lund, B., Ilver, D., Hamers, A., Gaastra, W., Karlsson, K. A. and Normark, S. (1990) 'Host-specificity of uropathogenic Escherichia coli depends on differences in binding specificity to Gal alpha 1-4Gal-containing isoreceptors.', *The EMBO journal*, 9(6), pp. 2001–10. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1693334 (Accessed: 23 December 2018).

Su, Y., Wang, J., Huang, Z. and Xie, B. (2017) 'On-site removal of antibiotics and antibiotic resistance genes from leachate by aged refuse bioreactor: Effects of microbial community and operational parameters', *Chemosphere*, 178, pp. 486–495. doi: 10.1016/j.chemosphere.2017.03.063.

Sugahara, H., Yao, R., Odamaki, T. and Xiao, J. Z. (2017) 'Differences between live and heat-killed bifidobacteria in the regulation of immune function and the intestinal environment',

Beneficial Microbes, 8(3), pp. 463–472. doi: 10.3920/BM2016.0158.

Sütçü, M., Aktürk, H., Gülümser-Şişko, S., Acar, M., Erol, O. B., Somer, A., Bilgiç, B. and Salman, N. (2018) 'Granulomatous amebic encephalitis caused by acanthamoeba in an immuncompetent child', *The Turkish Journal of Pediatrics*, 60(3), p. 340. doi: 10.24953/turkjped.2018.03.019.

Sütterlin, H., Alexy, R. and Kümmerer, K. (2008) 'The toxicity of the quaternary ammonium compound benzalkonium chloride alone and in mixtures with other anionic compounds to bacteria in test systems with Vibrio fischeri and Pseudomonas putida', *Ecotoxicology and Environmental Safety*, 71(2), pp. 498–505. doi: 10.1016/j.ecoenv.2007.12.015.

Tadesse, E., Teshome, M., Merid, Y., Kibret, B. and Shimelis, T. (2014) 'Asymptomatic urinary tract infection among pregnant women attending the antenatal clinic of Hawassa Referral Hospital, Southern Ethiopia.', *BMC research notes*, 7, p. 155. doi: 10.1186/1756-0500-7-155.

Taher, E. E., Méabed, E. M. H., Abdallah, I. and Abdel Wahed, W. Y. (2018) 'Acanthamoeba keratitis in noncompliant soft contact lenses users: Genotyping and risk factors, a study from Cairo, Egypt', *Journal of Infection and Public Health*. Elsevier, 11(3), pp. 377–383. doi: 10.1016/J.JIPH.2017.09.013.

Tajbakhsh, E., Tajbakhsh, S. and Khamesipour, F. (2015) 'Isolation and Molecular Detection of Gram Negative Bacteria Causing Urinary Tract Infection in Patients Referred to Shahrekord Hospitals, Iran.', *Iranian Red Crescent medical journal*, 17(5), p. e24779. doi: 10.5812/ircmj.17(5)2015.24779.

Tawfik, A. F., Alswailem, A. M., Shibl, A. M. and Al-Agamy, M. H. M. (2011) 'Prevalence and Genetic Characteristics of TEM, SHV, and CTX-M in Clinical *Klebsiella pneumoniae*

Isolates from Saudi Arabia', *Microbial Drug Resistance*, 17(3), pp. 383–388. doi: 10.1089/mdr.2011.0011.

Tchesnokova, V., Aprikian, P., Kisiela, D., Gowey, S., Korotkova, N., Thomas, W. and Sokurenko, E. (2011) 'Type 1 Fimbrial Adhesin FimH Elicits an Immune Response That Enhances Cell Adhesion of Escherichia coli', *Infection and Immunity*, 79(10), pp. 3895–3904. doi: 10.1128/IAI.05169-11.

Thirapanmethee (2012) 'Abstract of Extended Spectrum β-Lactamases: Critical Tools of Bacterial Resistance', *Mahidol University Journal of Pharmaceutical Science*, 39(1), pp. 1–8. Available at: http://www.pharmacy.mahidol.ac.th/journal/journalabstract.php?jvol=39&jpart=1&jconnum=1 (Accessed: 10 April 2016).

Thomas, C. P., Moore, L. S. P., Elamin, N., Doumith, M., Zhang, J., Maharjan, S., Warner, M., Perry, C., Turton, J. F., Johnstone, C., Jepson, A., Duncan, N. D. C., Holmes, A. H., Livermore, D. M. and Woodford, N. (2013) 'Early (2008–2010) hospital outbreak of Klebsiella pneumoniae producing OXA-48 carbapenemase in the UK', *International Journal of Antimicrobial Agents*, 42(6), pp. 531–536. doi: 10.1016/j.ijantimicag.2013.08.020.

Thumbikat, P., Berry, R. E., Schaeffer, A. J. and Klumpp, D. J. (2009) 'Differentiation-induced uroplakin III expression promotes urothelial cell death in response to uropathogenic E. coli.', *Microbes and infection / Institut Pasteur*, 11(1), pp. 57–65. doi: 10.1016/j.micinf.2008.10.008.

Todd, C. D., Reyes-Batlle, M., Martín-Navarro, C. M., Dorta-Gorrín, A., López-Arencibia, A., Martínez-Carretero, E., Piñero, J. E., Valladares, B., Lindo, J. F. and Lorenzo-Morales, J. (2015) 'Isolation and Genotyping of *Acanthamoeba* Strains from Soil Sources from Jamaica,

West Indies', *Journal of Eukaryotic Microbiology*, 62(3), pp. 416–421. doi: 10.1111/jeu.12197.

Tóth, I., Hérault, F., Beutin, L. and Oswald, E. (2003) 'Production of cytolethal distending toxins by pathogenic Escherichia coli strains isolated from human and animal sources: establishment of the existence of a new cdt variant (Type IV).', *Journal of clinical microbiology*, 41(9), pp. 4285–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/12958258 (Accessed: 1 January 2019).

Tripathi, T., Abdi, M. and Alizadeh, H. (2013) 'Role of phospholipase A_2 (PLA_2) inhibitors in attenuating apoptosis of the corneal epithelial cells and mitigation of Acanthamoeba keratitis.', *Experimental eye research*. NIH Public Access, 113, pp. 182–91. doi: 10.1016/j.exer.2013.05.021.

Tsiatsiou, O., Iosifidis, E., Katragkou, A., Dimou, V., Sarafidis, K., Karampatakis, T., Antachopoulos, C., Orfanou, A., Tsakris, A., Drossou-Agakidou, V. and Roilides, E. (2015) 'Successful management of an outbreak due to carbapenem-resistant Acinetobacter baumannii in a neonatal intensive care unit', *European Journal of Pediatrics*, 174(1), pp. 65–74. doi: 10.1007/s00431-014-2365-8.

Tsujimoto, H., Gotoh, N. and Nishino, T. (1999) 'Diffusion of macrolide antibiotics through the outer membrane of Moraxella catarrhalis.', *Journal of infection and chemotherapy : official journal of the Japan Society of Chemotherapy*, 5(4), pp. 196–200. doi: 10.1007/s101569900025.

Turk J Med Sci, Sefa C. Sailik, Üffet Palabiyikoúlu, J. S. B. (2000) 'Analysis of Methicillin Resistant Staphylococcus aureus Isolates by Polyacrylamide Gel Electrophoresis in an Intensive Care Unit of Übni-Sina Hospital', *T†BÜTAK*, 367(371). Available at: http://citeseerx.ist.psu.edu/viewdoc/summary?doi=10.1.1.530.5567 (Accessed: 11

November 2015).

Turner, P. J. (2005) ‘Extended-Spectrum β-Lactamases’, *Clinical Infectious Diseases*, 41(s4), pp. S273–S275. doi: 10.1086/430789.

Tzeng, Y.-L., Datta, A., Kolli, V. K., Carlson, R. W. and Stephens, D. S. (2002) ‘Endotoxin of Neisseria meningitidis Composed Only of Intact Lipid A: Inactivation of the Meningococcal 3-Deoxy-D-Manno-Octulosonic Acid Transferase’, *Journal of Bacteriology*, 184(9), pp. 2379–2388. doi: 10.1128/JB.184.9.2379-2388.2002.

Valat, C., Auvray, F., Forest, K., Métayer, V., Gay, E., Peytavin de Garam, C., Madec, J.-Y. and Haenni, M. (2012) ‘Phylogenetic grouping and virulence potential of extended-spectrum-β-lactamase-producing Escherichia coli strains in cattle.’, *Applied and environmental microbiology*, 78(13), pp. 4677–82. doi: 10.1128/AEM.00351-12.

Valenza, G., Nickel, S., Pfeifer, Y., Eller, C., Krupa, E., Lehner-Reindl, V. and Höller, C. (2014) ‘Extended-Spectrum-β-Lactamase-Producing Escherichia coli as Intestinal Colonizers in the German Community’, *Antimicrobial Agents and Chemotherapy*, 58(2), pp. 1228–1230. doi: 10.1128/AAC.01993-13.

Vanlangenakker, N., Vanden Berghe, T., Krysko, D. V, Festjens, N. and Vandenabeele, P. (2008) ‘Molecular mechanisms and pathophysiology of necrotic cell death.’, *Current molecular medicine*, 8(3), pp. 207–20. Available at: http://www.ncbi.nlm.nih.gov/pubmed/18473820 (Accessed: 12 December 2018).

Vatcheva-Dobrevska, R., Mulet, X., Ivanov, I., Zamorano, L., Dobreva, E., Velinov, T., Kantardjiev, T. and Oliver, A. (2013) ‘Molecular Epidemiology and Multidrug Resistance Mechanisms of *Pseudomonas aeruginosa* Isolates from Bulgarian Hospitals’, *Microbial Drug Resistance*, 19(5), pp. 355–361. doi: 10.1089/mdr.2013.0004.

Vázquez, R. F., Daza Millone, M. A., Pavinatto, F. J., Herlax, V. S., Bakás, L. S., Oliveira, O. N., Vela, M. E. and Maté, S. M. (2017) 'Interaction of acylated and unacylated forms of E. coli alpha-hemolysin with lipid monolayers: a PM-IRRAS study', *Colloids and Surfaces B: Biointerfaces*, 158, pp. 76–83. doi: 10.1016/j.colsurfb.2017.06.020.

Vejborg, R. M., Hancock, V., Schembri, M. A. and Klemm, P. (2011) 'Comparative Genomics of Escherichia coli Strains Causing Urinary Tract Infections', *Applied and Environmental Microbiology*. American Society for Microbiology, 77(10), pp. 3268–3278. doi: 10.1128/AEM.02970-10.

Venier, A. G., Talon, D., Patry, I., Mercier-Girard, D. and Bertrand, X. (2007) 'Patient and bacterial determinants involved in symptomatic urinary tract infection caused by Escherichia coli with and without bacteraemia', *Clinical Microbiology and Infection*, 13(2), pp. 205–208. doi: 10.1111/j.1469-0691.2006.01586.x.

Visvesvara, G. S., Moura, H. and Schuster, F. L. (2007) 'Pathogenic and opportunistic free-living amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea', *FEMS Immunology and Medical Microbiology*, 50(1), pp. 1–26. doi: 10.1111/j.1574-695X.2007.00232.x.

Waksman, G. and Hultgren, S. J. (2009) 'Structural biology of the chaperone–usher pathway of pilus biogenesis', *Nature Reviews Microbiology*, 7(11), pp. 765–774. doi: 10.1038/nrmicro2220.

Waldroup, A. L., Beers, K. L., Cook, P. E., Dell, E. A., Odglen, R., Baker, R. A., Coleman, C. W., Smith, B. A. and Maingi, B. W. (2010) 'The Effects of Cetylpyridinium Chloride (Cecure CPC Antimicrobial) on ® 1 Campylobacter Spp. on Raw Poultry: A Review', *International Journal of Poultry Science*, 9(4), pp. 305–308. Available at: http://emedicine.medscape.com/article/ (Accessed: 15 December 2018).

Wallace-Gadsden, F., Johnson, J. R., Wain, J. and Okeke, I. N. (2007) 'Enteroaggregative Escherichia coli related to uropathogenic clonal group A.', *Emerging infectious diseases*. Centers for Disease Control and Prevention, 13(5), pp. 757–60. doi: 10.3201/eid1305.061057.

Walther-Rasmussen, J. and Høiby, N. (2006) 'OXA-type carbapenemases.', *The Journal of antimicrobial chemotherapy*, 57(3), pp. 373–83. doi: 10.1093/jac/dki482.

Wang, G., Clark, C. G. and Rodgerst, F. G. (2002) 'Detection in Escherichia coli of the genes encoding the major virulence factors, the genes defining the O157:H7 serotype, and components of the type 2 Shiga toxin family by multiplex PCR', *Journal of Clinical Microbiology*, 40(10), pp. 3613–3619. doi: 10.1128/JCM.40.10.3613-3619.2002.

Waters, V. L. and Crosa, J. H. (1991) 'Colicin V virulence plasmids.', *Microbiological reviews*, 55(3), pp. 437–50. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1943995 (Accessed: 24 December 2018).

Waxman, D. J., Yocum, R. R. and Strominger, J. L. (1980) 'Penicillins and cephalosporins are active site-directed acylating agents: evidence in support of the substrate analogue hypothesis.', *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, 289(1036), pp. 257–71. Available at: http://www.ncbi.nlm.nih.gov/pubmed/6109322 (Accessed: 11 December 2018).

Weber, D. J., Tolkoff-Rubin, N. E. and Rubin, R. H. (1984) 'Amoxicillin and Potassium Clavulanate: An Antibiotic Combination Mechanism of Action, Pharmacokinetics, Antimicrobial Spectrum, Clinical Efficacy and Adverse Effects', *Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy*, 4(3), pp. 122–133. doi: 10.1002/j.1875-9114.1984.tb03333.x.

Weekers, P. H., Engelberts, A. M. and Vogels, G. D. (1995) ‘Bacteriolytic activities of the free-living soil amoebae, Acanthamoeba castellanii, Acanthamoeba polyphaga and Hartmannella vermiformis.’, *Antonie van Leeuwenhoek*, 68(3), pp. 237–43. Available at: http://www.ncbi.nlm.nih.gov/pubmed/8572682 (Accessed: 13 December 2018).

Wenzler, E., Fraidenburg, D. R., Scardina, T. and Danziger, L. H. (2016) ‘Inhaled Antibiotics for Gram-Negative Respiratory Infections.’, *Clinical microbiology reviews*. American Society for Microbiology Journals, 29(3), pp. 581–632. doi: 10.1128/CMR.00101-15.

Whitfield, C. and Roberts, I. S. (1999) ‘Structure, assembly and regulation of expression of capsules in Escherichia coli.’, *Molecular microbiology*, 31(5), pp. 1307–19. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10200953 (Accessed: 31 December 2018).

WHO (2014) *ANTIMICROBIAL RESISTANCE Global Report on Surveillance*. Available at: http://apps.who.int/iris/bitstream/handle/10665/112642/?sequence=1 (Accessed: 21 December 2018).

Wickramasinghe, N. H., Xu, L., Eustace, A., Shabir, S., Saluja, T. and Hawkey, P. M. (2012) ‘High community faecal carriage rates of CTX-M ESBL-producing Escherichia coli in a specific population group in Birmingham, UK’, *Journal of Antimicrobial Chemotherapy*. Oxford University Press, 67(5), pp. 1108–1113. doi: 10.1093/jac/dks018.

Wiles, Kulesus and Mulvey (2008) ‘Origins and virulence mechanisms of uropathogenic Escherichia coli.’, *Experimental and molecular pathology*, 85(1), pp. 11–9. doi: 10.1016/j.yexmp.2008.03.007.

Wiles, T. J., Kulesus, R. R. and Mulvey, M. A. (2008) ‘Origins and virulence mechanisms of uropathogenic Escherichia coli’, *Experimental and Molecular Pathology*, 85(1), pp. 11–19. doi: 10.1016/j.yexmp.2008.03.007.

Wilson, M. (2005) *Microbial Inhabitants of Humans: Their Ecology and Role in Health and Disease*. Cambridge University Press. Available at: https://books.google.com/books?id=q9Qx9oRKO_kC&pgis=1 (Accessed: 25 May 2015).

Winkler, M. L., Papp-Wallace, K. M., Taracila, M. A. and Bonomo, R. A. (2015) 'Avibactam and inhibitor-resistant SHV β-lactamases.', *Antimicrobial agents and chemotherapy*. American Society for Microbiology Journals, 59(7), pp. 3700–9. doi: 10.1128/AAC.04405-14.

Wlodkowic, D., Skommer, J. and Darzynkiewicz, Z. (2009) 'Flow Cytometry-Based Apoptosis Detection', in *Methods in molecular biology (Clifton, N.J.)*, pp. 19–32. doi: 10.1007/978-1-60327-017-5_2.

Worgall, S., Martushova, K., Busch, A., Lande, L. and Crystal, R. G. (2002) 'Apoptosis Induced by Pseudomonas aeruginosa in Antigen Presenting Cells Is Diminished by Genetic Modification with CD40 Ligand', *Pediatric Research*, 52(5), pp. 636–644. doi: 10.1203/00006450-200211000-00006.

Wragg, R., Harris, A., Patel, M., Robb, A., Chandran, H. and McCarthy, L. (2017) 'Extended spectrum beta lactamase (ESBL) producing bacteria urinary tract infections and complex pediatric urology', *Journal of Pediatric Surgery*, 52(2), pp. 286–288. doi: 10.1016/j.jpedsurg.2016.11.016.

Wright, K. J., Seed, P. C. and Hultgren, S. J. (2007) 'Development of intracellular bacterial communities of uropathogenic *Escherichia coli* depends on type 1 pili', *Cellular Microbiology*, 9(9), pp. 2230–2241. doi: 10.1111/j.1462-5822.2007.00952.x.

Wu, X.-R., Kong, X.-P., Pellicer, A., Kreibich, G. and Sun, T.-T. (2009) 'Uroplakins in urothelial biology, function, and disease.', *Kidney international*. NIH Public Access, 75(11),

pp. 1153–1165. doi: 10.1038/ki.2009.73.

Xia, J., Gao, J. and Tang, W. (2016) ‘Nosocomial infection and its molecular mechanisms of antibiotic resistance’, *BioScience Trends*, 10(1), pp. 14–21. doi: 10.5582/bst.2016.01020.

Xian, Y., Dong, H., Wu, Y., Guo, X., Hou, X. and Wang, B. (2016) ‘QuEChERS-based purification method coupled to ultrahigh performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) to determine six quaternary ammonium compounds (QACs) in dairy products’, *Food Chemistry*. Elsevier, 212, pp. 96–103. doi: 10.1016/J.FOODCHEM.2016.05.151.

Xu, W., Li, Y. and Fan, C. (2018) ‘Different loci and mRNA copy number of the increased serum survival gene of *Escherichia coli*’, *Canadian Journal of Microbiology*. NRC Research Press, 64(2), pp. 147–154. doi: 10.1139/cjm-2017-0363.

Yagi, S., Schuster, F. L. and Bloch, K. (2007) ‘Demonstration of Presence of Acanthamoeba Mitochondrial DNA in Brain Tissue and Cerebrospinal Fluid by PCR in Samples from a Patient Who Died of Granulomatous Amebic Encephalitis’, *Journal of Clinical Microbiology*, 45(6), pp. 2090–2091. doi: 10.1128/JCM.02435-06.

Yamamoto, S., Nakano, M., Terai, A., Yuri, K., Nakata, K., Nair, G. B., Kurazono, H. and Ogawa, O. (2001) ‘The presence of the virulence island containing the usp gene in uropathogenic Escherichia coli is associated with urinary tract infection in an experimental mouse model.’, *The Journal of urology*, 165(4), pp. 1347–51. Available at: http://www.ncbi.nlm.nih.gov/pubmed/11257714 (Accessed: 30 December 2018).

Yan, J. X., Wait, R., Berkelman, T., Harry, R. A., Westbrook, J. A., Wheeler, C. H. and Dunn, M. J. (2000) ‘A modified silver staining protocol for visualization of proteins compatible with matrix-assisted laser desorption/ionization and electrospray ionization-mass spectrometry.’,

Electrophoresis, 21(17), pp. 3666–72. doi: 10.1002/1522-2683(200011)21:17<3666::AID-ELPS3666>3.0.CO;2-6.

Yoo, K.-T. and Jung, S.-Y. (2012) 'Effects of Mannose on Pathogenesis of Acanthamoeba castellanii', *The Korean Journal of Parasitology*, 50(4), pp. 365–369. doi: 10.3347/kjp.2012.50.4.365.

Yousuf, F. A., Siddiqui, R. and Khan, N. A. (2013) 'Acanthamoeba castellanii of the T4 genotype is a potential environmental host for Enterobacter aerogenes and Aeromonas hydrophila.', *Parasites & vectors*, 6, p. 169. doi: 10.1186/1756-3305-6-169.

Yousuf, F. A., Yousuf, Z., Iqbal, J., Siddiqui, R., Khan, H. and Khan, N. A. (2014) 'Interactions of neuropathogenic Escherichia coli K1 (RS218) and its derivatives lacking genomic islands with phagocytic Acanthamoeba castellanii and nonphagocytic brain endothelial cells.', *BioMed research international*, 2014, p. 265424. doi: 10.1155/2014/265424.

Yu, H. S., Hwang, M. Y., Kim, T. O., Yun, H. C., Kim, T. H., Kong, H. H. and Chung, D. I. (1999) 'Phylogenetic relationships among Acanthamoeba spp. based on PCR-RFLP analyses of mitochondrial small subunit rRNA gene.', *The Korean journal of parasitology*, 37(3), pp. 181–8. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10507226 (Accessed: 30 December 2018).

Zaman, V., Zaki, M. and Manzoor, M. (1999) 'Acanthamoeba in human faeces from Karachi.', *Annals of tropical medicine and parasitology*, 93(2), pp. 189–91. Available at: http://www.ncbi.nlm.nih.gov/pubmed/10474646 (Accessed: 30 December 2018).

Zamani, K., Emami, A., Bazargani, A. and Moattari, A. (2015) 'Phenotypic and molecular characterization of CTX-M extended-spectrum beta-lactamase-producing Escherichia coli isolates in Shiraz, Iran', *Revista da Sociedade Brasileira de Medicina Tropical*, 48(4), pp.

479–482. doi: 10.1590/0037-8682-0315-2014.

Zamora, A., Henderson, H. and Swiatlo, E. (2014) ‘Acanthamoeba encephalitis: A Case Report and Review of Therapy.’, *Surgical neurology international*. Wolters Kluwer -- Medknow Publications, 5, p. 68. doi: 10.4103/2152-7806.132239.

Zarei, A. R., Bagheri Sadeghi, H. and Abedin, S. (2013) ‘Selective cloud point extraction for the spectrophotometric determination of cetylpyridinium chloride in pharmaceutical formulations.’, *Iranian journal of pharmaceutical research : IJPR*, 12(4), pp. 671–7. Available at: http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=3920716&tool=pmcentrez&rendertype=abstract (Accessed: 3 April 2016).

Zeng, X. and Lin, J. (2013) ‘Beta-lactamase induction and cell wall metabolism in Gram-negative bacteria.’, *Frontiers in microbiology*, 4, p. 128. doi: 10.3389/fmicb.2013.00128.

Zhang, C., Cui, F., Zeng, G., Jiang, M., Yang, Z., Yu, Z., Zhu, M. and Shen, L. (2015) ‘Quaternary ammonium compounds (QACs): A review on occurrence, fate and toxicity in the environment’, *Science of The Total Environment*, 518–519, pp. 352–362. doi: 10.1016/j.scitotenv.2015.03.007.

Zheng, X., Uno, T., Goto, T., Zhang, W., Hill, J. M. and Ohashi, Y. (2004) ‘Pathogenic Acanthamoeba Induces Apoptosis of Human Corneal Epithelial Cells’, *Japanese Journal of Ophthalmology*, 48(1), pp. 23–29. doi: 10.1007/s10384-003-0018-y.

Zhou, H., Pisitkun, T., Aponte, A., Yuen, P. S. T., Hoffert, J. D., Yasuda, H., Hu, X., Chawla, L., Shen, R.-F., Knepper, M. A. and Star, R. A. (2006) ‘Exosomal Fetuin-A identified by proteomics: a novel urinary biomarker for detecting acute kidney injury.’, *Kidney international*. NIH Public Access, 70(10), pp. 1847–57. doi: 10.1038/sj.ki.5001874.

Zhu, W., Smith, J. W. and Huang, C.-M. (2010) 'Mass Spectrometry-Based Label-Free Quantitative Proteomics', *Journal of Biomedicine and Biotechnology*, 2010, pp. 1–6. doi: 10.1155/2010/840518.

Zowawi, H. M., Balkhy, H. H., Walsh, T. R. and Paterson, D. L. (2013) '-Lactamase Production in Key Gram-Negative Pathogen Isolates from the Arabian Peninsula', *Clinical Microbiology Reviews*, 26(3), pp. 361–380. doi: 10.1128/CMR.00096-12.

CONFERENCES

List of authors/Title/Type of attendance	**Conference**	**Location**	**Date**
Participant	The Essex Biomedical Sciences Institute (EBSI) Conference 2015.	Colchester Hospital University, UK	1/7/2015
Qumsani, Alaa Talal; Alsam, Selwa Z W "Role of ESBL+ E. coli and Acanthamoeba in urinary tract infections". 14th Annual Graduate Forum 2015, School of Biological Sciences, University of Essex, Colchester, UK.	14th Annual Graduate Forum 2015	University of Essex, Colchester, UK	16/9/2015
Participant	Institute of Biomedical Science (IBMS) Congress 2015	Birmingham, UK	18/9/2015
Qumsani, Alaa Talal; Alsam, Selwa Z W "Role of *Acanthamoeba* and OXA-48 producing *E. coli* bacteria in Urinary Tract Infections". The first Saudi Scientific Forum at King Fahad Academy 2017, London, UK	The first scientific forum of scientific society for Saudi students in the UK	London, UK	1/4/2017
Participant	Breaking the Barriers: Interdisciplinary research and careers	Colchester, UK	25/4/2017
Participant	School of Biological Sciences: Research Conference	Colchester, UK	22/6/2017
Qumsani, Alaa Talal; Alsam, Selwa Z W **"**Endosymbiosis between ESBL positive *E. coli* and *Acanthamoeba* in UTIs**".** Institute of Biomedical Science (IBMS) Congress 2017-Birmingham, UK.	Institute of Biomedical Science (IBMS) Congress 2017	Birmingham, UK	24-27/ 9/2017
Qumsani, Alaa Talal; Alsam, Selwa Z W **"**The Inhibitory Effects Of Cetylypyridinium Chloride On Urinary Tract-Associated Pathogens**".** Healthcare Infection Society (HIS) Conference 2018-Liverpool, UK.	Healthcare Infection Society (HIS) Conference 2018	Liverpool, UK	26-28/11/2018