AsPac J. Mol. Biol. Biotechnol. 2019
Vol. 27 (1) : 84-94

Inoculation of fowlpox viruses coexpressing avian influenza H5 and chicken IL-15 cytokine gene stimulates diverse host immune responses

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Received 27th November 2018 / Accepted 4th February 2019

Abstract. Fowlpox virus (FWPV) has been used as a recombinant vaccine vector to express antigens from several important avian pathogens. Attempts have been made to improve vaccine strains induced-host immune responses by coexpressing cytokines. This study describes the construction of recombinant FWPV (rFWPV) strain FP9 and immunological responses in specific-pathogen-free (SPF) chickens, co-expressing avian influenza virus (AIV) H5 of A/Chicken/Malaysia/5858/2004, and chicken IL-15 cytokine genes. Expression of H5 (50 kD) was confirmed by western blotting. Anti-H5 antibodies, which were measured by the haemagglutinin inhibition test, were at the highest levels at Week 3 post-inoculation in both rFWPV/H5- and rFWPV/H5/IL-15-vaccinated chickens, but decreased to undetectable levels from Week 5 onwards. CD3+/CD4+ or CD3+/CD8+T cell populations, assessed using flow cytometry, were significantly increased in both WT FP9- and rFWPV/H5-vaccinated chickens and were also higher than in rFWPV/H5/IL-15- vaccinated chickens, at Week 2. Gene expression analysis using real time quantitative polymerase chain reaction (qPCR) demonstrated upregulation of IL-15 expression in all vaccinated groups with rFWPV/H5/IL-15 having the highest fold change, at day 2 (117±51.53). Despite showing upregulation, fold change values of the IL-18 expression were below 1.00 for all vaccinated groups at day 2, 4 and 6. This study shows successful construction of rFWPV/H5 co-expressing IL-15, with modified immunogenicity upon inoculation into SPF chickens.

Keywords: avian influenza virus, fowlpox virus, haemagglutinin, interleukin-15, interleukin-18

INTRODUCTION

Since the late 1980s, recombinant FWPVs (rFWPV)s based on attenuated FWPV strains have been developed to express antigens from several important avian pathogens, including: avian influenza virus (AIV; (Qian *et al.,* 2012)), Newcastle disease virus (NDV; (Sun *et al.,* 2008) and Marek's disease virus (MDV; (Lee *et al.,* 2003). rFWPVs expressing haemagglutinin (HA) H5 protein of AIV (rFWPV/H5), particularly derived from A/Turkey/Ireland/83 (H5N9), or A/Goose/Guangdong/96 (H5N1), have been used in South East Asia as vaccines against highly pathogenic avian influenza (HPAI) H5N1. Despite this preventive measure, HPAI H5N1 is

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still a major concern due to its ongoing, sporadic re-emergence. The need to boost existing eradication efforts to limit the spread and occurrence of the outbreak has prompted development of several strategies to improve readily available avian influenza vaccines. We describe here one such strategy: to co-express host cytokines from rFWPV/H5.

In mice, recombinant vaccinia virus (rVACV) co-expressing gp160 of human immunodeficiency virus (HIV) and human interleukin 15 (hIL-15) has been shown to provide a stronger and more enduring response than rVACV expressing gp160 alone (Oh *et al.,* 2003). Integration of hIL-15 into rVACV Wyeth strain or Modified VACV Ankara (MVA) resulted in better survival (Perera *et al.,* 2007) and enhanced *in vivo* viral clearance (Zielinski *et al.,* 2010) in vaccinated athymic nude mice upon intranasal challenge with virulent VACV strain Western Reserve, or intravenous challenge with monkeypox virus strain Zaire 79, respectively. Enhanced CD4 and CD8 T cell memory responses, along with reduction in lung mycobacterial load in lungs, was also observed in mice infected with Bacille Calmette-Guérin (BCG), supplemented with IL-7 and IL-15 recombinant proteins, but not IL-1, IL-6 or interferon (IFN)-α (Singh *et al.,* 2010). In mice model, it has also been shown that IL-15 offers potent antiviral effects against rVACV coexpressing IL-15, with high dependency on the presence of NK cells and IFNs (Foong *et al.,* 2009).

Almost all of the chicken cytokines that have been investigated are Th1-like. In a rare avian study, in ovo plasmid DNA vaccination against an intestinal coccidial parasite, *Eimeria acervulina,* using coccidial gene 3-1E coexpressed with chicken IL-15, was shown to induce higher serum antibody levels than immunization with 3-1E alone. Following challenge with the homologous parasite, chickens vaccinated with 3-1E plus IL-15 showed a significant decreased in oocyst shedding and had an increased body weight, compared to chickens vaccinated with 3-1E alone (Lillehoj *et al.,* 2005). Similar results were obtained whether the construct was given subcutaneously (Min *et al.,* 2001) or intramuscularly (Ma *et al.,* 2013).

Studies with rFWPV coexpressing HA from AIV H5N1 and chicken IL-18 (Chen *et al.,* 2011; Mingxiao *et al.,* 2006) or IL-6 (Qian *et al.,* 2012) have been described. The results showed that all chickens vaccinated with rFWPV/H5/IL-18 exhibited reduced virus shedding and replication (Chen *et al.,* 2011), and had higher levels of cellular immunity (Mingxiao *et al.,* 2006), compared to rFWPV/H5 alone. Study of the effect of chIL-15 coexpression by rFWPV/H5 in chickens, as reported here, is novel.

MATERIALS AND METHODS

Ethical approval. All animal experiments performed in this study were in accordance with the ethical standards of the local Institutional Animal Care and Use Committee (IACUC) of Universiti Putra Malaysia (UPM) with reference number UPM/FPV/PS/3.2.1.551/AUP-R72.

Viruses and cells. The initial stock of parental FP9 was from M.A. Skinner laboratory (Imperial College London, UK). The development of FP9 via 438 serial passages of the wild-type fowlpox virus HP-1, followed by plaque purification, has been described (Laidlaw and Skinner, 2004). Chicken embryonic fibroblast (CEFs) used in this study were cultured in 2% newborn bovine serum (NBBS) in DMEM media (both from Gibco).

Construction of recombinant plasmids. Previously cloned and sequenced cDNA encoding full-length H5 of influenza strain A/Ch/Malaysia/5744/2004 *(Balasubramaniam et al.,* 2011) was amplified by PCR with primers H5-F: 5'-ATCGGATATCATGGAGAAAATAGTG C-3' and H5-R: 5'-GACTGATATCTTAAA TGCAAATTCTGC-3', introducing *EcoRV* sites as underlined. Sequence encoding the pentabasic peptide motif at the protease cleavage site of H5 was replaced with threonine (T) using mutagenic primers S(2-F): 5'-CAAAGAGAGACAAGAGG ATTATTTGGAGCTATAG-3' and S(1-R): 5'-CAAATAATCCTCTTGTCTCTCTTTGAGG GCTATTTC-3'.

The assembled amplicon was inserted into the *SmaI* site of *lac Z*-selectable, FWPV expression/recombination vector pEFL29 (Qingzhong *et al.,* 1994), downstream of a copy of the vaccinia virus p7.5 early/late promoter. The

chicken IL-15 gene (supplied by the late Prof. Dr. Pete Kaiser from the then Institute for Animal Health, Compton, UK) was inserted downstream of a synthetic/hybrid promoter in vector pEF*gpt*12S, before being subcloned into vector pPC1.X (Abd Razak, 2011). Positive transformants were grown in LB broth (15 mL) supplemented with appropriate antibiotic(s) (750 μg) at 37°C overnight. The culture (0.5 μL) was used to provide templates for analytical PCR. The reaction mixture for a small scale PCR verification contained 10X PCR buffer (2 μL; Sigma), JumpStart *Taq* DNA polymerase (0.5 U; Sigma), dNTPs (0.5 μL of 10 mM) and oligonucleotide primers (0.5 μL of each 10 μM stock), in a total volume of 20 μL. PCR was conducted in 2 steps; 4 cycles of 95°C for 3 min, 95°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min followed by 26 cycles of 95°C for 30 s, 59°C for 30 s, and 7°C for 1.5 min. Final extension was operated at 72°C for 10 minutes. Verification of H5 integration into FWPV was carried out using H5-F and H5-R primers, while primers pPC1.X-F: 5'-ATGAAAAATAGTACCACTATGG-3' and IL-15R: 5'-ACAGAGTTTTGTAAAGGTTATACA GAGG-3' were used to screen for rFWPV/H5 carrying IL-15 gene.

Recombination/transfection, selection and purification of recombinant viruses. The detailed protocol for recombination/transfection has been described (Laidlaw and Skinner, 2014), with minor modifications i.e. replacing 199 media with DMEM (Gibco), and polyfect with lipofectin (Thermo Fisher Scientific), of the same volume. Successful recovery of rFWPV/H5 carrying a *LacZ* gene from pEFL29 into FP9 was demonstrated by blue plaques upon X-Gal overlay (at final concentration of 0.4 mg/mL) on day 4 post-transfection. Further screening for rFWPV/H5 carrying chIL-15 was done by mycophenolic acid (MPA) selection of *gpt* gene and spontaneous resolving of the *gpt* gene by a second crossover event, as described previously (Laidlaw *et al.,* 1998). The recombinant protein lysates were prepared by infecting CEFs, with rFWPV/H5 at a multiplicity of infection (MOI) of 3, for 48 hours. The cell pellet was subjected to 15% SDS-PAGE. The electro-transferred nitrocellulose membrane (GE Healthcare) was incubated with a goat polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab62587, Abcam, USA) with the final concentration 1 μg/μL, for 1 hour. The membrane was developed using a commercial kit using the chromogenic substance, WesternBreeze (Invitrogen).

Immunofluorescence antibody test (IFAT) was performed using 80% confluent CEFs. Cells were either infected with viruses at 0.3 MOI, or left uninfected (negative control). The infection was left overnight in 2% NBBS DMEM medium, before incubation with a rabbit polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab70077, Abcam, USA) with the final concentration 1 μg/μL, for 2 hours. After three washes with PBS, cells were incubated with fluorescein-labelled secondary antibodies for 1 hour. Slides were viewed under a fluorescent microscope (model Leica DMRA II).

Immunization of animals. One-day old specific pathogen-free (SPF) chickens were inoculated subcutaneously with 10^5 plaque forming unit (PFU) of parental FWPV FP9 (WT FP9), rFWPV/H5 or rFWPV/H5/IL-15, diluted in PBS to a total volume of 100 μL, at the scruff of the neck, using a 27-G needle. One control group was mock-treated with 100 μL of PBS. Nine chickens were assigned for each group. Blood sampling (for serum) of each chicken was done on a weekly basis. At Weeks 2 and 5, whole blood (0.2 mL) of each chicken in each group of nine was sampled and pooled into 3 groups (0.6 mL in total), for CD4+ and CD8+ lymphocyte isolation, followed by flow cytometry analysis. As for IL-15 and IL-18 gene expression analysis, immunization of 105 PFU of aforementioned vaccine groups was done on 14-days old SPF chickens; twelve chickens for each group. At every two consecutive days' post immunization, RNA was extracted from the spleens (four from each group) and processed for qPCR.

Serological tests. Haemagglutination inhibition (HI) tests were performed in U-bottomed 96-well microtitre plates using 4 HA units/25 μL of H5N2 virus strain A/Malaysia/Duck/8443/04 (Veterinary Research Institute Ipoh, Malaysia), and washed chicken erythrocytes (25 μL of 0.8% v/v). The antigen-antibody was incubated for 1 hour. HI titres were determined as the reciprocal

of the highest serum dilution that completely inhibited haemagglutination.

Immunophenotyping analysis. Fresh, non-coagulated chicken whole blood was diluted to 1 mL using cold PBS and was carefully layered on 2 mL Ficoll-Paque PLUS (GE Healthcare). Isolation of peripheral blood mononuclear cells (PBMC) was done by following the standard Ficoll-Paque PLUS protocol. Approximately 10^6 cells were incubated with mouse anti-chicken CD8a-PerCP-Cy5-conjugated (1 μg/mL), CD3-PE-conjugated (0.5 μg/mL) and CD4-FITC-conjugated (0.5 μg/mL) monoclonal antibodies (all from Southern Biotech), prior to analysis using a BD FACSCalibur flow cytometer (Becton Dickinson, San Jose, CA, USA).

Quantitative real time polymerase chain reaction (qPCR). The total RNA from chicken spleens was harvested using TRIzol reagent (Ambion) according to manufacturer's recommendations. The extracted RNA was reverse-transcribed using Script cDNA synthesis kit (Jena Bioscience) in a total volume of 20 μL containing 2.5 μM primers, 1X Script reverse transcriptase (RT) buffer, 500 μM dNTP, 5 μM DTT stock, 40 units RNAse inhibitor, 100 units Script RT and 5 μg RNA template. The reaction mix was incubated at 42°C for 10 minutes followed by 50°C for 60 minutes. Primer sequences for cytokines IL-15 and IL-18, and a housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH), were designed from public databases (Brisbin *et al.,* 2010; Cai *et al.,* 2009), as shown in Table 1. The qPCR amplification was performed according to the KAPA SYBR FAST qPCR kit (KAPA Biosystem) using Bio-Rad CFX96 real-time system. The data was imported into the analysis module of the Bio-Rad CFX Manager. The expression of GAPDH gene were used as the qPCR normalization standards. All results are reported as delta-delta CT (ΔΔCT), relative to the control group.

Statistical analysis. Data variations between groups were analysed by one-way ANOVA or paired-samples T test using SPSS (Version 15) software. Results were expressed as the mean ± standard error of the mean (SE). P values less than 0.05 were considered statistically significant in all cases.

Table 1. Forward and reverse primer sequences used for qPCR.

Primer name	Primer sequence (5' to 3')	GenBank accession number
IL-15 - F	CGAGGCTTGTACCGCAATGT	AF139097
IL-15 - R	GCCATCCCCAGCATCTTGT	
IL-18 - F	ACAAGGAATGTTCTTGGCCTTT	NM_204608
IL-18 - R	CTTCATCTTCTCTCGGCAGTTTC	
GAPDH - F	CTACACACGGACACTTCAAG	NM_204305
GAPDH - R	ACAAACATGGGGGCATCAG	

F, forward; R, reverse; IL, interleukin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

RESULTS

PCR amplification of H5 gene. The HPAIV haemagglutinin (HA) gene of the H5 virus contains multiple basic amino acids, arginine and lysine, that allow cleavage by ubiquitous proteases (furin and PC6) (Horimoto *et al.,* 1994). To maintain compatibility with recombinant, killed H5N1 influenza vaccines (J. Wood, personal communication), and to reduce any potential biosafety issues, the pentabasic peptide motif (underlined) at the protease cleavage site of H5, S-P-Q-R-E-R-R-R-K-K-R was removed and replaced with threonine (T), leaving a monobasic arginine (R) at the site (S-P-Q-R-E-T-R). The H5-F and S(1-R) primers generated the first H5 fragment (1036 bp), while primers H5-R and S(2-F) generated the second H5 fragment (684 bp). Full length mutated H5 gene (1695 bp), was obtained through PCR overlap extension mutagenesis (Figure 1a).

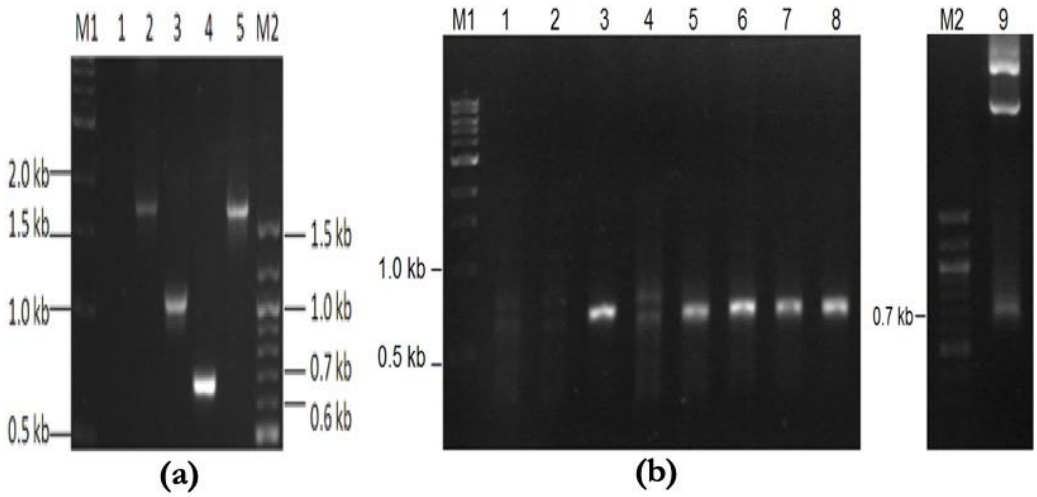

Figure 1. (a) PCR amplification of H5 gene upon removal of polybasic sequence. Lane 1: Negative control; Lane 2: Full length HA H5 gene (1707 bp); Lane 3: Fragment 1 (1036 bp) and Lane 4: Fragment 2 (684 bp) were generated using mutagenic primers S(2-F) and S(1-R); Lane 5: Re-assembled full length H5 sequence, lacking the polybasic region (1695 bp) was obtained using PCR overlap extension mutagenesis. (b) Verification for the integration of the IL-15 gene into rFWPV/H5 by PCR after genomic DNA extraction, with exclusive amplicon of 700 bp which corresponds to the positive control. Lanes 1-8: Genomic DNA of recombinant clones rFWPV/H5 after second homologous recombination. Lane 9: IL-15 plasmid positive control. M1: 1 kb ladder marker (New England Biolabs); M2: 100 bp ladder marker (New England Biolabs). The DNA bands were observed in 1% (w/v) agarose gel.

Verification of H5-recombinant fowlpox viruses coexpressing chicken IL-15. Upon successful transfection, the recombinant clones were verified for presence of the inserted H5 gene by PCR of extracted FWPV genomic DNA (data not shown). Positive recombinants (rFWPV/H5) were subjected to second homologous recombination of vector pPC1.X carrying chicken cytokine gene IL-15 at a second non-essential site, the PC-1 (*fpv030*) homology region. The cytokine expression cassettes in pPC1.X/IL-15 were previously confirmed by restriction digests and sequencing (data not shown). Screening of positive recombinant viruses (rFWPV/H5/IL-15) was done using primers external and internal to the inserted genes (the latter resulting in PCR products exclusively for recombinant clones) (Figure 1b). H5 protein expression was analysed by western blotting (Figure 2). A faint band at ~50 kD was observed for H5 recombinant, none for uninfected cell lysate and negative control (WT FP9). This is the first report on the size of H5 protein from strain A/Ch/Malaysia/5744/2004. Further analysis using IFAT detected fluorescent signals only for CEF infected with H5 recombinant, which indicates successful H5 protein expression. No reactivity was observed for uninfected or WT FP9-infected CEF (Figure 3).

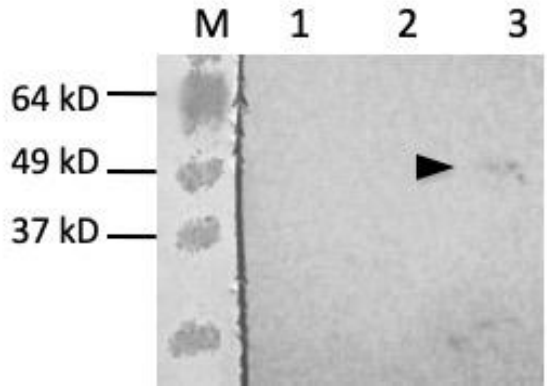

Figure 2. Western blot analysis of CEF cells infected with rFWPV/H5. Transferred nitrocellulose membrane was incubated with a goat polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab62587, Abcam, USA) with the final concentration 1 μg/μL, for 1 hour and separated on 12% SDS-PAGE. M: Benchmark™ Pre-stained Protein Ladder (Thermo Fisher Scientific); Lane 1: Uninfected CEF as negative control; Lane 2: CEF infected with WT FP9; Lane 3: CEF infected with rFWPV/H5.

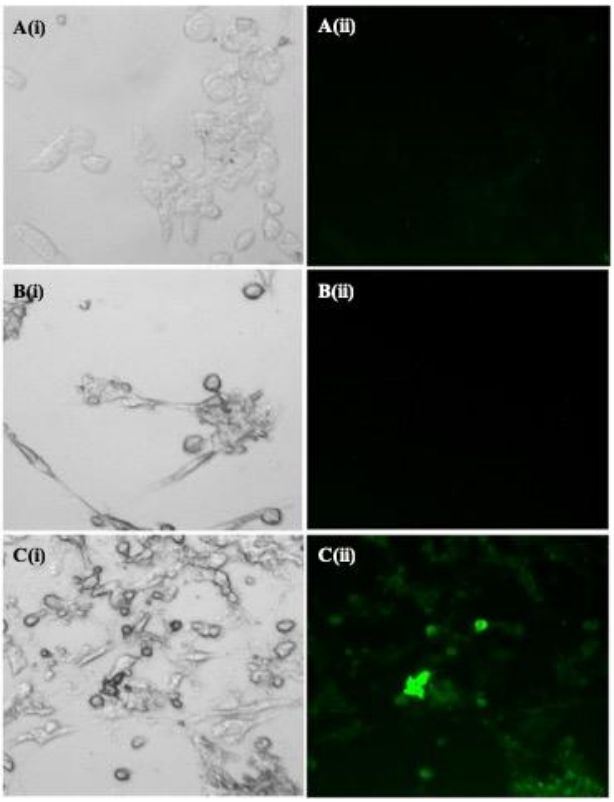

Figure 3. IFAT analysis for verification of H5 protein expression from rFWPV/H5. CEFs were (A) uninfected; (B) infected with WT FP9 as negative control; (C) infected with rFWPV/H5. Infected cells were incubated with a rabbit polyclonal primary antibody against haemagglutinin H5 (Cat. No. ab70077, Abcam, USA) with the final concentration 1 μg/μL. Observation was performed under visible light (ii) or UV light (ii). The images did not represent 80% of cell confluency due to repeated washing of the cells without fixation during procedure.

Haemagglutinin inhibition tests for chickens following rFWPV immunizations. None of the nine control chickens inoculated with PBS or WT FP9 showed any evidence of HI antibody responses. Mean HI titres, in $\log_2$, of all groups were calculated for general comparison (Table 2). H5 antibodies reached detectable levels in chickens vaccinated with rFWPV/H5/IL-15 one week earlier than those vaccinated with rFWPV/H5 but, thereafter, there was no significant difference between the two groups. Responses were highest at Week 3 in both groups of recombinant vaccine-treated chickens. However, the antibodies were undetectable based on HI tests that have been carried out from Week 5 onwards.

CD3+/CD4+ and CD3+/CD8+ T cells population following rFWPV immunizations. The levels of CD3+/CD4+ T cells in the control group remained relatively constant at Weeks 2 and 5. Samples from groups vaccinated with WT FP9 or rFWPV/H5/IL-15 demonstrated increases in CD3+/CD4+ T cell population levels over time, of 2.06 and 3.16 point percentages, respectively. The rFWPV/H5 vaccinated group showed a significantly higher CD3+/CD4+ T cell population relative to control at Week 2 ($P \leq 0.05$) but had returned to control levels by Week 5. No statistically significant difference in CD3+/CD4+ T cell levels was observed for other groups at either sampling point (Figure 4).

Animal experiments also revealed a relatively constant CD3+/CD8+ T cell population for control chickens. The same was true for the slight to somewhat higher levels observed in rFWPV/H5/IL-15-, WT FP9- and rFWPV-vaccinated birds (significant for WT- and rFWPV/H5- but not rFWPV/H5/IL-15-vaccinated birds), although a fall to control levels was observed in rFWPV/H5-vaccinated birds at Week 5.

Table 2. Mean of HI titre, $\log_2$, of sera from immunized chickens.

Vaccine group	HI titre						
	Weeks, post immunization						
	1	2	3	4	5	6	7
Control	ND	ND	ND	ND	ND	ND	ND
WT FP9	ND	ND	ND	ND	ND	ND	ND
rFWPV/H5	ND	ND	8.11±4.60	3.56±1.94	ND	ND	ND
rFWPV/H5/IL-15	ND	0.56±0.44	9.89±2.16	3.11±1.74	ND	ND	ND

ND indicates undetected titre. Each value represents the means±SE of nine birds.
No significant difference was observed between rFWPV/H5 and rFWPV/H5/IL-15 at any time point ($P \geq 0.05$).

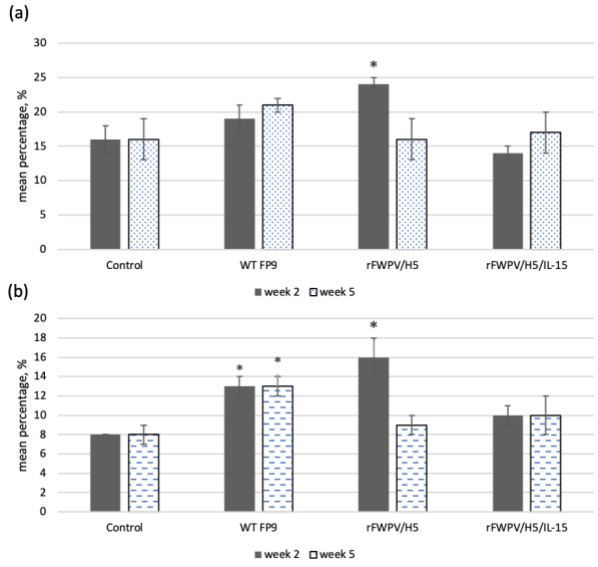

Figure 4. Immunophenotyping of CD3+/CD4 (a) and CD3+/CD8+ (b) lymphocytes from chickens after mock-treatment with PBS (control), or vaccination with WT FP9, rFWPV/H5 or rFWPV/H5/IL-15. Each value represents the mean percentages of T lymphocytes sub-population ± SE, from PBMC samples of nine chickens pooled in threes (n=3), sampled at Weeks 2 and 5. Significant differences between vaccinated and control groups (*), were determined by one-way ANOVA ($P \leq 0.05$). Significant differences within the same group at different points were determined by paired-samples T-test ($P \leq 0.05$).

Gene expression analysis of IL-15 and IL-18. IL-15 expression in all vaccinated groups showed upregulation on day 2, notably in those vaccinated with rFWPV/H5/IL-15, which overexpress chicken IL-15 (Figure 5). WT FP9- and rFWPV/H5-vaccinated birds expressed 7- to 19-fold more IL-15 than control birds; over-expression by rFWPV/H5/IL-15 boosted IL-15 levels to 120 fold more than control. Expression of IL-15 dropped to control levels by day 4, for all tested groups.

IL-18 expression was lower in all of the FWPV-infected groups (two to five fold lower for WT FP9- and rFWPV/H5-vaccinated groups, possibly up to ten fold lower for rFWPV/H5/IL-15) and this decreased expression was extended out to 6 days.

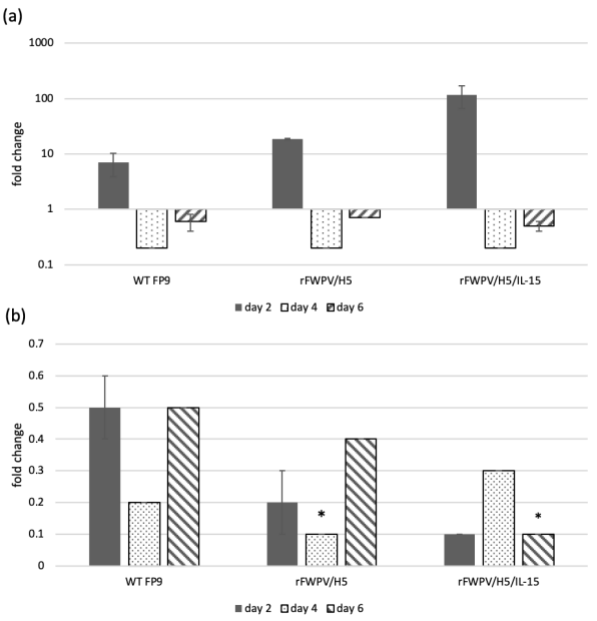

Figure 5. Relative expression level, by qPCR, of IL-15 (a) and IL-18 genes (b) in inoculated SPF chickens compared to control SPF chickens. The expression was expressed as fold change (2 log - ΔΔCT) to that of the unvaccinated controls after normalization of expression to GAPDH. The standard errors were calculated from the result of three replicates. Significant differences between vaccinated groups and WT FP9 (*) were determined by paired-samples T-test ($P \leq 0.05$).

DISCUSSION

The most important component of host immune response that confers protection in chickens against AIV is the humoral response against HA (Swayne, 2007). To achieve this, several different types of vaccines have been developed, e.g. inactivated AIV vaccines (Bublot *et al.,* 2007; Tian *et al.,* 2010), DNA vaccines (Lim *et al.,* 2012), and virus-like particles (Hendin *et al.,* 2017). In this study, a safe, lab-adapted FWPV-based vector expressing the H5 of AIV was modified to co-express a chicken IL-15 cytokine gene to test if it would enhance the host cell mediated immune response, which may be critical in clearance of AIV during infection (Foong *et al.,* 2009). However, we did not perform protective or challenge studies for AIV, nor did we evaluate protection against FWPV.

Vaccination with rFWPV/H5/IL-15 produced HA antibody titres comparable to

vaccination with rFWPV/H5. This finding contrasts with several studies conducted in mice, including that by Perera *et al.* (2007) which reported induction of two-fold higher VACV-neutralizing antibody titres in hIL-15-expressing recombinant VACV. The group also showed that recombinant VACV strain Wyeth, expressing five heterologous influenza virus genes, induced stronger neutralizing antibodies against AIV H5 when adjuvanted with hIL-15 (Poon *et al.,* 2009). The inconsistencies in HA antibody titres between our study and those by Perera *et al.* (2007) and Poon *et al.* (2009) might be due to the usage of heterologous (instead of a homologous) H5N2 virus strain A/Malaysia/Duck/8443/04 antigens against the H5 antibodies from our rFWPV recombinants. Heterologous antigens used to assay H5 antibodies induced by rFWPV were shown to produce either low (Taylor *et al.,* 1988), highest (Bublot *et al.,* 2010) or inconsistent (Swayne *et al.,* 2007) HI titres. Although these studies did not use homologous antigens, which might be more suitable for their HI testing, the results provide useful comparisons of HI antibody levels elicited by rFWPV/H5 and rFWPV/H5/IL-15. Several studies have shown rFWPV expressing H5 can provide complete or nearly complete protection against lethal challenge, even when achieving pre-challenge HI titres of as low as 3 $\log_2$ (Bublot *et al.,* 2010; Webster *et al.,* 1991). Post-vaccination protection of chickens against AIV may not be dependent entirely on HI antibodies but also on non-HI antibodies and possibly also on cell-mediated immunity.

rFWPV/H5/IL-15 did not increase CD4+ T cell populations, compared to rFWPV/H5, following vaccination. This finding is consistent with previous reports that IL-15 only has profound effects on the proliferation and survival of memory CD8+ T cells, not on CD4+ T cells (Marks-Konczalik *et al.,* 2000; Zhang *et al.,* 1998), although a significant increment of CD4+ T cell populations was observed in a DNA vaccine coexpressing H5 and chicken IL-15 genes (Lim *et al.,* 2012). It is not known whether inherent molecular patterns of, or immunomodulatory proteins expressed by, FWPV FP9 can influence IL-15 levels in vaccinated chickens. It has been reported that IL-15 can only activate CD4+ T cell proliferation when at high concentration presence (Kanegane and Tosato, 1996). Niedbala *et al.* (2002) showed that 2 to 4 fold higher concentrations of IL-15 are required to achieve optimal CD4+ T cell proliferation than to promote CD8+ T cell response.

The co-stimulatory effects of IL-15 on CD8 cells have been studied widely, especially with regard to proliferation and survival of memory CD8+ T cells. IL-15 has been found to directly stimulate purified CD8+ memory cells *in vitro* (Zhang *et al.,* 1998). Transgenic mice which constitutively expressed a significant level of IL-15 in the serum had higher numbers of memory CD8+ T cells (Marks-Konczalik *et al.*, 2000; Yajima *et al.,* 2002). In our study, chickens vaccinated with WT FP9 or rFWPVs showed low to moderate increase in levels of CD8+ T cells. The increases, at 1.6 to 2 fold, were significant for WT FP9 or rFWPV/H5 respectively but, at 1.25 fold increment, was insignificant from the rFWPV/H5/IL-15. These results suggest that FWPV enhances chicken CD8+ T cells stimulation and possibly that IL-15 has the opposite effect.

Although hIL-15 has been shown to stimulate CD8+ T cells population and promote the maintenance of CD8+ CD44hi memory T cells, the responsiveness of CD8+ T cells to IL-15 might depend on the cytokine background (Niedbala *et al.,* 2002; Oh *et al.,* 2003). Unfortunately, in this study, we did not measure the levels of IL-15, secreted by cells infected with an initial dose of 10^5 PFU rFWPV/H5/IL-15, in peripheral blood prior to flow analysis. Since a strong synthetic/hybrid promoter was used for IL-15 co-expression, levels of expression might have been inconsistent with generation of the desired immune responses.

We observed elevation of the CD4+ T cell population and sustained CD8+ T cell population from WT FP9 and rFWPV/H5/IL-15 inoculated groups. However, rFWPV/H5 inoculated group showed a consistent decreasing pattern for both T cells. By way of comparison, an *in vivo* study examining T cell populations in the peripheral blood of rhesus macaques treated with rhesus IL-15, where the level of CD4+ and CD8+ memory, but not naïve, T cells peaked at Weeks 1 to 2 and returned to baseline by Weeks 3 to 4 (Picker *et al.,* 2006).

Acting synergistically, IL-15 and IL-18 can

perpetuate Th1 responses (Gracie *et al.,* 1999) and enhance IL-12 stimulation of NK cell to produce IFN gamma (French *et al.,* 2006). A DNA vaccine co-expressing H5 and chicken IL-15, induced a significant increase in IL-15, but not IL-18, levels post-vaccination (Lim *et al.,* 2012). Our results are comparable, where enhanced expression of host IL-15 and reduced expression of host IL-18 are mediated directly by infection with WT FP9 or rFWPV/H5 co-expressing exogenous IL-15 (mediated by a strong synthetic poxvirus promoter).

The dramatic drop of IL-15 levels from day 2 to day 4 in all FWPV-infected groups might be due to clearance of these attenuated viruses by NK cells, their cytolytic activity potentially augmented by the ability of IL-15 (expressed endogenously by the host or exogenously by the recombinant FWPV) to enhance IFN expression and increase poxvirus clearance (Foong *et al.,* 2009). However, we cannot currently explain the concomitant drop in IL-18 mRNA expression during FWPV infection but FWPV appears to express one or more IL-18 binding proteins (Laidlaw and Skinner, 2004), which might reduce steady-state levels of circulating IL-18 in a similar manner to the host-encoded regulator IL-18BP (Harms *et al.,* 2017). It is possible therefore that the virus encodes additional mechanisms to down-regulate expression of IL-18 mRNA.

CONCLUSION

rFWPV/H5 and rFWPV/H5/IL-15 inoculated groups elicited the highest levels of anti-H5 antibodies at Week 3 post-inoculation. CD3+/CD4+ or CD3+/CD8+ T cell populations were significantly increased in both WT FP9- and rFWPV/H5-, higher than in rFWPV/H5/IL-15-vaccinated chickens, at Week 2 post-inoculation. IL-15 and IL-18 expressions were upregulated in all vaccinated groups at day 2 post-inoculation. These diverse immunogenicity findings may contribute to the limited exploration of chicken IL-15 in vaccine developments.

ACKNOWLEDGEMENTS

This study was supported by the Ministry of Energy, Science, Technology, Environment and Climate Change (MESTECC) with project number 02-01-04-SF1641, and the Ministry of Education through Institute of Bioscience, Higher Institution Centre of Excellence (IBS HICoE) with project number 6369101, from the Government of Malaysia. N.N.M. was funded by MyBrain15 of Ministry of Education, Malaysia, and Graduate Research Fellowship (GRF) of Universiti Putra Malaysia. M.A.S. and E.S.G. are supported by the Biotechnology and Biological Sciences Research Council via Strategic LoLa BB/K002465/1 ("Developing Rapid Responses to Emerging Virus Infections of Poultry, DRREVIP").

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