Enhancing control of virulent recombinant strains of laryngotracheitis virus using vaccination

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ABSTRACT

Infectious laryngotracheitis virus (ILTV) is an alphaherpesvirus that causes acute upper respiratory tract disease in chickens. Attenuated live ILTV vaccines are often used to help control the disease, but these vaccines have well documented limitations including natural recombination between different vaccine strains. Recently, two novel ILTV field strains (class 8 and 9 ILTV viruses) emerged in Australia due to natural recombination involving two distinct commercial ILTV vaccines. These recombinant field strains became dominant in important poultry producing areas and caused severe disease in commercial poultry flocks, showing that more options are needed to enable control of ILTV. The work described in this thesis investigated tools to better control disease due to ILTV.

Firstly, different commercial ILTV vaccines and a developmental candidate vaccine, glycoprotein G-deficient ILTV (ΔgG ILTV, registered as Vaxsafe ILT, Bioproperties Pty Ltd) were investigated for their ability to protect commercial broiler chickens against challenge with the virulent recombinant class 9 ILTV after drinking water vaccine delivery. All vaccines induced partial protection by direct (drinking-water) and indirect (contact) exposure when birds were subsequently challenged with the virulent class 9 challenge strain.

A vaccination and challenge study was then performed to determine the minimum effective dose of ΔgG ILTV that, when delivered by eye-drop to layer birds, would protect the birds from a robust challenge with class 9 ILTV. A dose of $10^{3.8}$ plaque

forming units per bird was the lowest dose capable of providing a high level of protection against challenge, as measured by clinical signs of disease, tracheal pathology and viral replication after challenge.

Finally, attempts were made to develop suitable tools to measure the level of immunity induced by ILTV vaccination. To this end, an ELISA that measures the amount of chicken interferon gamma (IFN-γ) was developed and used to quantitate IFN-γ production from splenocytes stimulated with control mitogens, or with ILTV antigen. The assay could detect IFN-γ released from chicken splenocytes after stimulation by concanavalin A. However, when splenocytes were incubated with semi-purified ILTV antigens *in vitro*, there was no increase in the level of ILTV specific IFN-γ production by splenocytes from ILTV infected birds, compared to uninfected birds. A number of potential avenues for further development of this assay were identified.

The work described in this thesis demonstrates that currently available vaccines and the new Vaxsafe ILT vaccine can be used to help control class 9 ILTV when delivered by drinking water. When delivered by eye-drop the Vaxsafe ILT vaccine candidate can induce a high level of protection against class 9 ILTV at a commercially feasible dose. Taken together, the results from this work lay the foundations on which a commercial vaccine may be developed, thereby offering the potential to provide producers with another important tool to help control ILTV.

Future development of a tool to measure protective immunity after vaccination is needed and would be a valuable addition to disease control programmes.

DECLARATION

This is to declare that all scientific work described was performed by me except where due acknowledgement has been made in the text and in the acknowledgement section. This thesis complies with The University of Melbourne requirements in being less than 100 000 words in length, exclusive of tables, figures and bibliographies.

Mesula Geloye Korsa

PREFACE

The work presented in this thesis was performed in the Melbourne Veterinary School, The Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Australia. The first results chapter presented in this thesis is directly from a published paper (see peer reviewed paper number 1, below), and the second results chapter is directly from a paper that is currently under review (see peer reviewed paper number 2, below). I have contributed 70% to each paper as a primary author, and 30% of each article was contributed by the co-authors. Details of these papers, and also conference presentations arising from my PhD studies, are provided below.

PEER REVIEWED ARTICLES

1. Korsa MG, Browning GF, Coppo MJ, Legione AR, Gilkerson JR, Noormohammadi AH, et al. Protection induced in broiler chickens following drinking-water delivery of live Infectious laryngotracheitis vaccines against subsequent challenge with recombinant field virus. PLoS One, 2015; 10: e0137719.

2. Korsa MG, Joanne M. Devlin, Carol A. Hartley, Glenn F. Browning, Mauricio J.C. Coppo, José A. Quinteros, et al., 2018. Determination of the

minimum effective dose for a glycoprotein-G-deficient infectious laryngotracheitis virus delivered via eye-drop route of vaccination in 7-day old SPF chickens. Submitted to PLoS One on 4/4/2018.

POSTERS PRESENTED AT CONFERENCES

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2. Korsa MG, Browning GF, Noormohammadi AH, Hartley C A, and Devlin JM. Development of a novel ILTV specific chicken interferon gamma enzyme linked immunosorbent assay, 42nd Annual International Herpesvirus Workshop, July 29-August 2, 2017; Ghent, Belgium, (Poster).

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LIST OF ABBREVIATIONS

°C	degrees Celsius
µg	microgram
µL	microliter
AGID	agar gel immunodiffusion
BAC	bacterial artificial chromosome
Bp	base pair
BSA-V	bovine serum albumin fraction V
$C0_2$	carbon dioxide
CAM	chorioallantoic membrane
CEK	chicken embryo kidney
CEL	chicken embryo liver
CEO	chicken embryo origin
ChIFGAM	chicken interferon gamma
CMI	cell-mediated immunity

Con A	concanavalin A
CPE	cytopathic effect
DIVA	Differentiating Infected from Vaccinated Animals
DMEM	Dulbecco’s Minimal Essential Medium
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
Dpc	day post challenge
Dpv	day post vaccination
ELISA	enzyme-linked immunosorbent assay
ELiSpot	enzyme-linked immunospot
FBS	foetal bovine serum
FPV	fowlpox virus
gB	glycoprotein B
gC	glycoprotein C
GCN	genome copy number

gD	glycoprotein D
gG	glycoprotein G
gI	glycoprotein I
gJ	glycoprotein J
h	hour
H&E	haematoxylin and eosin
HEPES	4-(2-hydroxyethly)-1-piperazineethanesulfonic acid
HVT	herpesvirus of turkey
I	ionomycin
IFA	indirect immunofluorescence antibody
IFN-γ	interferon gamma
ILT	infectious laryngotracheitis
ILTV	infectious laryngotracheitis virus
IR	internal repeat domain
LMH	leghorn male hepatoma

M	molar
Min	minute
mL	milliliter
mM	millimolar
NDV	Newcastle disease virus
ng	nanogram
nm	nanometer
ORF	open reading frame
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PBST	phosphate buffered saline plus Tween 20
PCR	polymerase chain reaction
PFU	plaque forming unit
pg	picogram
PMA	phorbol 12-myristate 13-acetate

qPCR	quantitative PCR
RFLP	restriction fragment length polymorphism
rLS	recombinant Lasota NDV
rpm	revolutions per minute
SD	standard deviation
SPF	specific pathogen free
TCO	tissue culture origin
UL	unique long region
US	unique short region
UV	ultraviolet
vCKBP	viral chemokine binding protein
w/v	weight per volume
x g	gravitational force
β-ME	betamercaptoethanol
ΔgG ILTV	glycoprotein G deficient ILTV

ΔORF C ILTV	ORF C deficient ILTV

1. Review of the Literature

1.1 Introduction

Infectious laryngotracheitis (ILT) is a highly contagious viral upper respiratory tract disease of chickens that causes significant economic losses in poultry industries worldwide (Bagust et al., 2000; Garcia et al., 2013). It is caused by infectious laryngotracheitis virus (ILTV), an alphaherpesvirus (Davison, 2010). The disease was first reported in the USA in 1925 (May and Tistler 1925) and it was then reported in Australia in 1935 (Seddon and Hart, 1935). Transmission is mainly horizontal, from clinically ill to healthy susceptible birds via direct contact, and can result in large outbreaks of disease (Devlin et al., 2011). Commercially available attenuated vaccines are often used to control the disease (Garcia et al., 2013; Gelenczei and Marty, 1964; Samberg et al., 1971). However, recently it has been shown that two distinct attenuated vaccine strains can recombine in the field and result in virulent recombinant viruses. Recombinant field strains, classified genotypically as class 8 and 9 viruses, emerged due to natural recombinant events between the genomes of existing Australian vaccines (class 1 ILTV) and the recently introduced European vaccine strain, Serva (class 7 ILTV) (Blacker et al., 2011; Lee et al., 2012). The recombinant class 9 ILTV has spread and caused outbreaks of disease in commercial poultry flocks, displacing the

historically predominant class 2 ILTV. The class 9 virus has increased potential for transmission and enhanced pathogenicity, consistent with its predominance in the field, compared to class 2 ILTV (Lee et al., 2014a). The ability of commercially available conventionally attenuated vaccines to protect chickens against challenge with the novel recombinant class 9 ILTV has not yet been studied.

Accurate discrimination of protected flocks from those not protected after vaccination is of paramount importance for successful disease control programs. The detection of antibodies in sera is commonly used to monitor the success of ILTV vaccination programs (Bauer et al., 1999; Coppo et al., 2013b; Rodríguez-Avila et al., 2008; Sander and Thayer, 1997; Shil et al., 2012). However, neither neutralizing nor mucosal antibodies are protective against ILTV (Fahey and York, 1990; Fulton et al., 2000). Therefore, antibody titres in the sera of vaccinated flocks do not necessarily reflect the level of protection after vaccination programs (Coppo et al., 2013b; Coppo et al., 2012; Fahey et al., 1983; Fahey and York, 1990). Despite this limitation, ILTV-specific antibody detection by ELISA is the only method available for screening flocks after ILTV vaccination (Coppo et al., 2013b). Therefore, a more accurate method for evaluation of the protective immune status of flocks or individual birds following vaccination would be useful to enhance disease control strategies.

Cell-mediated immunity (CMI) has been shown to be the most important component of the immune response in protective immunity against ILTV (Chen et al., 2011; Coppo et al., 2018; Fahey et al., 1984; Honda et al., 1994b). Interferon gamma (IFN-γ) plays an important role in the CMI response (Ariaans et al., 2008; Chen et al., 2011; Lambrecht et al., 2004; Smith et al., 2001; Yin et al., 2013). For some other important avian pathogens, such as Newcastle disease virus (NDV) and *Eimeria tenella*, the detection of IFN-γ production has been used as an indicator of CMI activity after vaccination (Ariaans et al., 2008; Lambrecht et al., 2004; Yin et al., 2013). There are different assay formats for the detection of IFN-γ production. Some of the T cell assays currently being used for the detection of CMI responses in other important avian pathogens include pathogen specific IFN-γ enzyme linked immunosorbent (IFN-γ-ELISA) and enzyme linked immunospot (IFN-γ-ELispot) assays. The detection of CMI responses to specific pathogens utilizing IFN-γ production as an indicator has also been well established in other food producing animal species (e.g. BOVIGAM® assay) (Rothel et al., 1992; Schiller et al., 2009; Wood and Jones, 2001). The kinetics of interferon gamma production have also been shown to have positive correlation with the level of protection induced against challenge with swine fever virus (Suradhat et al., 2001). However, for ILTV, quantification of vaccine induced protective CMI immune responses is limited by the lack of tools to conduct these assays. Therefore, there is a need to develop a sensitive assay to measure chicken cellular immune responses to ILTV vaccination,

1.2 Infectious laryngotracheitis virus

Infectious laryngotracheitis virus belongs to genus *Iltovirus* of the sub-family *Alphaherpesvirinae* within the family *Herpesviridae* (Davison et al., 2009). *Psittacid herpesvirus virus 1* (PsHV-1), the causative agent of Pacheco's disease of parrots, has also been placed within this same genus (Thureen and Keeler, 2006). ILTV is principally a pathogen of chickens, but pheasants, partridges and peafowl can also be infected (Crawshaw and Boycott, 1982). The virus is taxonomically designated *Gallid alphaherpesvirus 1* (GaHV-1) (Adams et al., 2016, Davison, 2010).

Infectious laryngotracheitis virus has a linear double stranded DNA genome that is 151,607 nucleotides in length. The genome contains unique long (UL) and unique short (US) regions, with the US region flanked by an inverted repeat (IR) and a terminal repeat (TR) region (Fuchs et al., 2007; Lee et al., 2011). Inversions of an internal part of the UL region (UL 22 to UL 44) (Garcia et al., 2013) and translocation of the UL 47 gene to the US region are also seen in the ILTV genome (Thureen and Keeler, 2006). Unlike other alphaherpesviruses, there is no homologue of UL 16 in ILTV (Fuchs et al., 2007). There are three origins of viral DNA replication, two *OriS* located within the inverted repeats and *OriL* located within the UL region (Garcia et al., 2013).

As with other herpesviruses, ILTV replication occurs in the nucleus of infected cells (Granzow et al., 2001). The initial attachment of the virus to host cell receptors is independent of chondroitin and heparan molecules, unlike other herpesviruses. Instead the virus directly interacts with a specific cellular receptor (Kingsley and Keeler Jr, 1999). Once in the nucleus of the host cell, viral replication begins, with cascades of transcription and protein expression occurring in a regulated and sequential order. Classically, three waves of transcription occur, resulting in the formation of messenger RNAs for three groups of polypeptides, designated α (immediate early, IE), β (early, E) and γ (late, L). Transcription is catalysed by cellular ribonucleic acid polymerase II (Costanzo et al., 1977). A recent study of transcript kinetics found that ILTV genes do not strictly adhere to this profile and have more “leaky” gene expression profiles. Thus, ILTV genes were re-classified as (immediate early, IE), (early, E), (early - late, E - L) and (late, L) based on the abundance of transcripts over the course of infection and their dependence on *de novo* protein synthesis or DNA replication (Mahmoudian et al., 2012). In this study, the transcript for ICP4 was the only one that accumulated in the presence of cycloheximide, confirming a previous report that ICP4 was the only ILTV IE gene (Veits et al., 2003b). Protein synthesis occurs in the cytoplasm, whereas transcription and replication of viral DNA occur within the nucleus (Garcia et al., 2013). The egress of viral progeny from infected cells occurs through exocytosis (Granzow et al., 2001).

The ILTV virion has the typical morphology of an alphaherpesvirus. The capsid is icosahedral in shape and the virion is composed of DNA core within a

nucleocapsid, which is surrounded by a tegument and then by a lipid envelope containing membrane associated proteins, particularly the viral glycoproteins (Davison et al., 2009). The glycoproteins are responsible for triggering both humoral and cell-mediated immune responses. Some of the glycoproteins, including glycoprotein B (gB) (Poulsen and Keeler, 1997), glycoprotein C (gC) (Kingsley and Keeler Jr, 1999), glycoprotein D (gD) (Spear and Longnecker, 2003), glycoprotein G (gG) (Devlin et al., 2006b; Devlin et al., 2008), and glycoprotein J (gJ) (Mundt et al., 2011), have been functionally characterized and shown to be immunogenic. Deletion of ILTV genes encoding the glycoproteins has resulted in the attenuation of viral virulence. Some gene-deletion ILTV strains, including gG- and open reading frame (ORF) C-deficient strains, have showed promise as potential vaccine candidates (García, 2016).

The virus can be propagated in the allantoic cavity and on the chorioallantoic membrane (CAM) of embryonated chicken eggs. It can also be grown in variety of avian cell cultures, including chick embryo liver (CEL), chick embryo kidney (CEK), and chicken kidney cell cultures (Schnitzlein et al., 1994). The leghorn male hepatoma (LMH) cell line, which was derived from a chemically induced male chicken liver tumour (Kawaguchi et al., 1987) can also be used for propagation of ILTV.

1.3 Immunological responses to ILTV

As in mammals, the innate and adaptive immune systems provide defence against infection in birds (Erf, 2004; Sharma, 1999). The innate immune system components are always present or are rapidly mobilised following an encounter with a pathogen (Coppo et al., 2013a; Netea et al., 2016). The mucous lining, as well as the epithelial barrier, of the respiratory tract serves as a first line of defence against ILTV by forming physical barriers to infection (Coppo et al., 2013b; Sharma, 1999). Inflammatory responses are also crucial innate immune responses controlling viral replication and contributing to the subsequent pathogenesis of ILT. Natural killer cells and macrophages constitute another important part of the innate immune response in chickens (Schat, 1994). These cells are generally thought to lack the capacity to remember encounters with pathogens. However, recent studies in other animal species have indicated that cells of the innate immune system have some capacity to enhance responsiveness after re-encounter with pathogens, a mechanism described as ‘trained immunity’ or ‘innate immune memory’. This immunological memory is mainly driven by epigenetic changes and has a short lifespan compared to that of standard adaptive immunological memory (Netea et al., 2016). Understanding the importance of this ‘trained immunity’ during ILTV infection may open a new avenue for elucidating the pathogenesis of this virus, which may be helpful in enhancing disease control strategies.

In contrast, adaptive immune responses develop relatively slowly, but are antigen specific because antigen receptor re-arrangements result in long lasting immunological memory (Netea et al., 2016). The responses of the adaptive immune system involve humoral and cell mediated immunity. The humoral arm of the adaptive immune response is mediated by antibodies and other soluble macromolecules present in extracellular fluids. Antigen specific antibodies are generated after surface immunoglobulins serve as receptors for antigen recognition by B cells (Sharma, 1999). However, the humoral immune response is not protective against ILTV. Neither circulating neutralising antibodies (Bauer et al., 1999; Fahey et al., 1983; Shil et al., 2012) nor mucosal antibodies are effective in inducing protective immunity against ILTV (Fahey and York, 1990). Maternally derived antibodies are also unable to protect against infection with ILTV. An early study showed that chicks hatched from hyper-immune hens were as susceptible to ILT as those hatched from unvaccinated hens (Garcia et al., 2013). Reports about the effect of maternal antibodies on the efficacy of ILTV vaccines differ. One study has reported that the efficacy of an ORF C-deficient ILTV vaccine strain was reduced in the presence of maternal antibodies after *in ovo* vaccination (García et al., 2016). This interference was reportedly overcome in birds that were *in ovo* inoculated with the ORF C-deficient strain and then revaccinated at day 8 of age via spray (García et al., 2016). A more recent study evaluated the ability of NDV LaSota strain-vectored ILTV gB and gD recombinant vaccines to induce protective immunity against ILTV following intranasal and intraocular vaccine delivery and reported no significant interference by maternal antibody with the efficacy of this viral vectored ILTV vaccine (Yu et al., 2017). The

differences could be explained by the different vaccine constructs, ages of vaccination and/or routes of vaccine delivery.

Fahey et al. demonstrated adoptive transfer of immunity against ILTV between inbred birds using both hyper-immune and memory spleen cells. But the transfer of non-immune splenocytes, or thymocytes and bursal cells from immune animals, failed to confer immunity (Fahey et al., 1984). Honda et al. reached a similar conclusion from a study that showed bursectomized chickens developed resistance to ILTV following intravenous inoculation with immune splenic and peripheral blood mononuclear cells (Honda et al., 1994a). The increasing availability of research tools and reagents for studying chicken immune responses are expected to help in development of a better understanding of the mechanisms involved in CMI responses against ILTV (Coppo et al., 2013b). The use of next generation sequencing technology (RNA-seq) has yielded promising results in the characterization of antigen-specific immunological responses in chickens (Hamzić et al., 2016; Luo et al., 2014). This method can help in understanding immunological responses involved in combatting invading pathogens during either natural infection or induction of protective immunity after vaccination and may aid the design of better disease control strategies.

1.4 Infectious laryngotracheitis clinical disease

1.4.1 Transmission and pathogenesis

Chickens are the primary host of ILTV, and all ages are susceptible to infection. The virus enters the host mainly through the upper respiratory tract and ocular routes via direct contact with infected birds or fomites. Rarely, the virus can also enter the host via ingestion of contaminated feeds (Garcia et al., 2013). Following entry, incubation periods vary from 6 to 12 days in naturally exposed birds, but from only 2 to 4 days in experimentally infected birds, where the virus is inoculated directly into the trachea. Irrespective of the portal of entry, the epithelium of the trachea and larynx is affected, and the primary site of viral replication is the tracheal epithelium (Bagust et al., 2000; Coppo et al., 2013a). Infected chickens excrete the virus and transmit it to susceptible birds (Coppo et al., 2011; Garcia et al., 2013). One study has estimated the reproductive ratio (R_0, the average number of secondary infectious cases from a typical infectious case) of ILTV infection to be 2.43 (Devlin et al., 2011). Although infected birds are the main source of infection, ILTV can also remain viable outside of the host on external surfaces, and in the internal organs of adult beetles and their larvae, as demonstrated by qPCR and viral isolation in embryonated eggs, suggesting beetles infected with live vaccine virus may be a source of infection with ILTV (Ou and Giambrone, 2012). It can also remain viable in biofilms on water lines (Ou et al., 2011). Chickens that have received live attenuated vaccines can transmit the vaccine virus to naïve birds during the early phase of infection, when the virus is actively replicating in the tracheal mucosa (Coppo et al., 2012; Rodríguez-Avila et al., 2008). Both vaccine and wild type strains of ILTV can establish life-long

latent infections in the infected birds (Bagust, 1986; Williams et al., 1992). The site of latency has been shown to be the trigeminal ganglion (TG) (Williams et al., 1992). Latently infected birds excrete the virus following stressful events, such as rehousing or the onset of lay (Hughes et al., 1989). Wind-borne ILTV transmission between the poultry farms has also been reported to be significant (Johnson et al., 2005). There is no evidence for egg-transmission of ILTV or for shedding of the virus on shells of eggs laid by infected hens (Garcia et al., 2013).

1.4.2 Clinical signs

The clinical signs of ILT can vary from very mild to severe. The mild form can be indistinguishable from other mild respiratory diseases of chickens (Garcia et al., 2013). The characteristic clinical signs during the mild form of disease include a slow onset of illness, unthriftiness, nasal discharge, watery ocular discharge, conjunctivitis, and a drop in egg production. The morbidity is high, but the mortality varies with the virulence of the infecting viral strain (Fuchs et al., 2007; Garcia et al., 2013). During the severe form of the disease, additional clinical signs can include gasping, coughing, expectoration of bloody mucus and dyspnoea. Clots of blood may be observed on the floor and walls of poultry houses, as well as sudden deaths due to asphyxia (Fuchs et al., 2007). The severity of clinical signs induced by ILTV depends on the route of exposure, the virulence of the viral strain involved, and the initial viral load that was inoculated (Garcia et al., 2013; Kirkpatrick et al., 2006).

1.4.3 Diagnosis

The severe form of ILT may be diagnosed based on the clinical signs and post-mortem findings. In most cases, laboratory tests, including histological examination, detection of the agent and detection of agent-specific host immune responses are necessary to confirm the diagnosis (Garcia et al., 2013).

1.4.3.1 Histopathological examination

Diagnosis by histological examination is based on detection of pathognomonic intra-nuclear inclusion bodies and syncytial cells in tracheal tissues stained with Giemsa or haematoxylin and eosin (Garcia et al., 2013). The inclusion bodies are the classical Cowdry type A inclusions of herpesviruses, but they may be present for only 3 – 5 days after infection. In severe cases, where most infected cells have detached from the tracheal lining, inclusions may be seen in intact cells among the cellular debris in the lumen of the trachea (Pirozok et al., 1957). Tissues other than the trachea, such as the spleen, the bursa of Fabricieus and the caecal tonsils, can also be considered for histopathological examination, as ILTV can spread beyond the respiratory tract, the primary tissue where viral replication occurs (Kirkpatrick et al., 2006; Wang et al., 2013).

1.4.3.2 Detection of agent

Different techniques can be used to detect ILTV in clinical samples. These include agar gel immunodiffusion (AGID), immunoperoxidase (IP) staining of tissue sections, direct electron microscopy (EM), antigen capture ELISA, polymerase chain reaction (PCR) assays and PCR in combination with restriction fragment length polymorphism (RFLP) (Alexander and Nagy, 1997; Kirkpatrick et al., 2006; Mahmoudian et al., 2011; Shil et al., 2015; Shil et al., 2012; York and Fahey, 1988). Virus may be isolated on the CAM of embryonated chicken eggs at 9 - 12 days of incubation, or in susceptible cell cultures. Viral growth causes pocks on the CAM and syncytial formation in the cultured cells. However, viral isolation is time consuming and multiple passages may be needed to detect the presence of virus (Garcia et al., 2013). After isolation, the virus can be identified and genotyped using PCR and DNA sequencing, or PCR combined with RFLP (Blacker et al., 2011; Oldoni et al., 2008; Oldoni et al., 2009; Shil et al., 2015). A reverse RFLP (RRFLP) technique has also been described to distinguish the vaccine strain genotype from that of wild type virus. Briefly, the RRFLP assay based on the difference in the cycle threshold number obtained after *Alw*I or *Ava*I digestion and that obtained with undigested DNA. Samples with a Ct after *Alw*I digestion greater than or equal to that obtained with undigested DNA and a Ct value after *Ava*I digestion less than or equal to that obtained with undigested DNA are the vaccine strain genotype (CEO type commercial USA vaccine), whereas those that yield results the opposite of this are defined as having the wild type genotype (Callison et al., 2009). Recently, a quantitative real time PCR assay

using Taqman technology that can differentiate wild-type and the glycoprotein G-deficient candidate vaccine strain has been described (Shil et al., 2015). ILTV can also be detected in the clinical samples such as feather-pulps, feather shafts and tracheal exudates using PCR (Davidson et al., 2015; Davidson et al., 2018; Davidson et al., 2016).

1.4.3.3 Antibody detection

Different techniques can be utilized to detect anti-ILTV antibodies in chicken serum. These include ELISAs, viral neutralization (VN), AGID, and indirect immunofluorescence antibody (IFA) tests (Bagust et al., 2000). AGID and indirect immunofluorescence tests are rarely used nowadays, while ELISAs are the method of choice to detect ILTV antibody (Coppo et al., 2013b). ELISA-based detection of antibody has greater sensitivity than VN, and a comparable level of sensitivity to IFA. It is also more suited to testing large numbers of sera (Bauer et al., 1999). Detection of antibodies against glycoprotein I (gI) pre- and post-challenge in chickens vaccinated with an HVT-ILT recombinant viral vaccine expressing ILTV gI has been reported to be capable of differentiating between vaccinated and infected birds (Vagnozzi et al., 2012). An ELISA that detects antibodies specific for recombinant gG of ILTV has also been shown to have potential as a companion diagnostic tool in conjunction with use of the gG-deficient ILTV vaccine (Shil et al., 2012). This is an important feature as it allows discrimination between infected and vaccinated animals (DIVA approach).

1.4.3.4 Detection of cellular immune responses

To date, relatively few methods have been used to measure CMI responses to ILTV. These include antigen specific lymphocyte proliferation assays, immunohistochemical staining, and flow cytometry assays (Chen et al., 2011; Devlin et al., 2010). However, these methods are time consuming and can also lack sensitivity. The production of pathogen-induced IFN-γ has been shown to reflect T cell functionality to specific pathogens. IFN-γ release by T cells following antigen recall activation can be detected using different assay formats. The use of ELISA and ELISpot assays to detect CMI activities to vaccination has also been described in poultry (Ariaans et al., 2008; Lambrecht et al., 2004; Yin et al., 2013). Detection of cellular immune responses to ILTV using a similar quantitative assay has not been reported. The development of an ILTV-specific IFN-γ ELISA assay to detect cellular immune responses to ILTV vaccination would be helpful for identifying correlates of protection and enhancing disease control programs.

1.5 ILT control strategies and challenges

1.5.1 Biosecurity measures

Routine biosecurity procedures can be effective in the prevention of ILTV entry into a flock and spread between flocks. These procedures include restriction of contact between vaccinated birds or birds that have recovered from infection with wild type virus and birds that are unvaccinated, area biosecurity procedures in which all growers and companies avoid sharing labour and equipment, standard hygiene practices and control of access to the flock of animals such as rodents, dogs and wild birds (which may act as vectors for spread) (Dufour-Zavala, 2008; Garcia et al., 2013). The importance of geographic information systems in the management of ILT outbreaks has also been described (Dufour-Zavala, 2008).

1.5.2 Vaccination

1.5.2.1 Commercial attenuated live vaccines

Initially, immunization of chickens against ILTV was achieved by application of tracheal scrapings from infected birds to the cloaca of susceptible birds. Although this successfully elicited local inflammatory responses and protected chickens from upper respiratory disease, viral shedding from upper respiratory tract was not reduced (Gibbs, 1934), limiting the suitability of the approach. Later, virulent ILTV strains were attenuated by sequential passage in cell culture or embryonated chicken eggs. Commercially available ILTV vaccines have been attenuated either by multiple passages in embryonated eggs (chicken embryo

origin, or CEO, vaccines) (Samberg et al., 1971) or by continuous passage in tissue culture (tissue culture origin, or TCO, vaccines) (Gelenczei and Marty, 1964). Initially, these vaccines were tested and licensed for inoculation of individual birds but, as a result of the expansion of the poultry industry, mass vaccination via drinking water or spray has become the most common method for vaccine delivery (García, 2016). However, administration via drinking water risks incomplete flock immunity (Fulton et al., 2000; Robertson and Egerton, 1981), while delivery by spraying can result in adverse reactions (Clarke et al., 1980).

Protocols for vaccination vary, but can involve a single vaccination at 2 weeks of age or older for the induction of flock immunity that lasts for 15 - 20 weeks (Bagust et al., 2000; Garcia et al., 2013). In multi-age layer flocks, different protocols are used, with chickens vaccinated twice before the onset of egg production. The vaccine can be delivered at 7 weeks of age, followed by booster dose at 15 weeks of age. This protocol has been shown to confer superior protection against challenge and results in effective control of ILT in multi-age layer flocks (Fulton et al., 2000). Prophylactic vaccination against ILT is often not required in broilers because of their short growth cycle and the stringent biosecurity measures used for most flocks. However, vaccination may still be required when an outbreak has occurred in a nearby flock or when the disease has previously occurred on the farm. Many factors can affect the outcome of vaccination with attenuated vaccines, including the dose, the vaccination schedule and the route of administration (Garcia et al., 2013). Birds receiving suboptimal dose of vaccine

as commonly reported after drinking water vaccine delivery may develop incomplete immunity and such unprotected animals serve as reservoirs for future disease outbreaks (Fulton et al., 2000). Spray vaccination can also result in adverse side effects such as development of severe respiratory signs (Clarke et al., 1980; Fulton et al., 2000) and *in ovo* vaccine delivery may fail to elicit substantial levels of protection due to immunity systems immaturity (Gimeno et al., 2011).

In Australia, three traditionally attenuated CEO ILTV vaccines are used to control ILT. These are the SA2 and A20 strains (Zoetis Australia) and the Serva strain (MSD Animal Health). The A20 vaccine strain was derived from the SA2 vaccine strain by further attenuation through 20 additional passages in chick embryos and is considered a safer vaccine suitable for use in young chickens and broilers. The SA2 vaccine strain is less attenuated and is recommended for use as a booster vaccine in layers after initial vaccination with A20 (Bagust and Johnson, 1995; Coppo et al., 2013b; Purcell and Surman, 1974).

Traditionally attenuated ILTV vaccines have well documented limitations. These include regaining virulence following *in vivo* passage, transmission from vaccinated to unvaccinated birds, and the establishment of latency (Coppo et al., 2012; García, 2016; Guy et al., 1991; Neff et al., 2008; Oldoni et al., 2008; Williams et al., 1992). A landmark study by Guy et al. (1991) clearly illustrated that after sequential passage in specific pathogen free (SPF) chickens, both TCO

and CEO ILTV vaccines regained virulence levels comparable with that of a wild type strain (Guy et al., 1991). The exact mechanism(s) that leads to the increase in virulence of ILT vaccine viruses remains to be determined (Guy et al., 1991). One possible explanation of increased virulence following bird-to-bird transmission of vaccine virus is the dose dependency of disease. During bird-to-bird transmission of vaccine virus, viral dose is not controlled, unlike during vaccination. It is possible that birds receive much higher doses during bird-to-bird transmission than they receive during vaccination and this might explain the increased pathogenicity. Lifelong latent infections can be established in vaccinated birds (Bagust, 1986; Williams et al., 1992). This latent infection can be reactivated following stress (Hughes et al., 1989). Therefore, the use of such vaccines is only recommended in areas where ILT is endemic (Coppo et al., 2013b).

Natural recombination events between two distinct attenuated live vaccine strains have resulted in the generation of novel virulent recombinant ILTVs. Evidence from whole genome sequence analysis of the live attenuated vaccine strains in Australia, along with the genome of the newly emerged strains, have confirmed that the new viruses (class 8 and 9 ILTVs) emerged as a result of recombination between the European-origin Serva vaccine strain and the Australian vaccine strains (SA2 and A20). The newly emerged strains have spread and are now the viruses predominantly responsible for outbreaks of disease in important poultry producing areas of Australia, including in Victoria and New South Wales (Blacker et al., 2011; Lee et al., 2012).

Despite these limitations, traditionally attenuated ILTV vaccines are often considered to be the most effective vaccines for control of ILT (García, 2016). The immunity induced by these vaccines can protect against challenge with virulent virus from a distinct ILTV lineage, highlighting the fact that traditionally attenuated live vaccines remain useful tools for control of ILT in poultry industries (Lee et al., 2014a). The effectiveness of traditionally attenuated ILTV vaccines may be attributable to their ability to trigger the entire repertoire of antigen-specific host immune responses which may result in the development of effective immune responses against a pathogen. Results obtained from efficacy studies using live attenuated vaccines of human alphaherpesviruses, have showed superior protection against infection compared to other vaccine types including subunit and recombinant vaccines (Stanfield and Kousoulas, 2015).

1.5.2.2 Inactivated vaccines

Inactivated vaccines were developed to overcome the limitations of attenuated live vaccines. They were made using either inactivated whole ILTV (Barhoom et al., 1986) or affinity-purified glycoproteins (York and Fahey, 1991). Although such vaccines are capable of protecting chickens from challenge with ILTV, the expense involved in vaccine production and the high doses needed make them unsuitable for immunization of large scale commercial flocks (Fuchs et al., 2007).

1.5.2.3 Commercial recombinant virally vectored vaccines

The frequency of outbreaks of disease associated with CEO ILTV vaccines have prompted efforts to develop recombinant viral vectored vaccines. Expression of immunogenic antigens of ILTV in viral vectors has generated effective recombinant ILT vaccines. Two different viral vectors have been used for commercially available ILTV vaccines, herpesvirus of turkeys (HVT) and fowl pox virus (FPV). The FPV vectored vaccine carries the ILTV UL 27 and UL 32 genes, which encode gB and a membrane-associated protein, respectively (Davison et al., 2006). Another FPV-vectored vaccine, co-expressing NDV genes and ILTV gB has been developed in China. Under laboratory conditions, this candidate vaccine induced a comparable level of protection against challenge to that induced by live attenuated vaccines measured by morbidity and mortality rates as well as challenge viral isolation in CAM (Sun et al., 2008). The HVT vectored vaccine carries the US 6 and US 7 genes of ILTV, which encode gD and gI, respectively (Vagnozzi et al., 2012). Although HVT vectored vaccines were initially registered for subcutaneous and transcutaneous applications, the increasing incidence of ILT outbreaks in the United States of America has encouraged the broiler industry to deliver HVT vectored vaccines *in ovo* (Davison et al., 2006; García, 2016; Johnson et al., 2010). FPV vaccines were registered for wing web administration in breeders and subcutaneous inoculation in day-old commercial layers (García, 2016; Johnson et al., 2010; Vagnozzi et al., 2012).

Currently, none of the recombinant viral vectored products are in use in Australian poultry (Agnew-Crumpton et al., 2016).

Several studies have evaluated the protective efficacy of commercial recombinant viral vectored vaccines when administered *in ovo* or subcutaneously (Davison et al., 2006; Johnson et al., 2010; Sun et al., 2008; Vagnozzi et al., 2012). It was speculated that recombinant virally vectored ILTV vaccines may meet the need for better control of ILT (Bagust and Johnson, 1995). However, in general, it has been found that the levels of protection induced after vaccinating chickens with the vectored vaccines are inferior to those elicited by administration of traditionally attenuated live vaccines, particularly the CEO vaccines (Sun et al., 2008; Vagnozzi et al., 2012). Field studies have also found that in regions where there is a high level of challenge the commercial recombinant virally vectored vaccines have been incapable of inducing full protection against disease (Johnson et al., 2010). However, one study has reached a different conclusion, reporting that vaccination with a commercial recombinant virally vectored vaccine induced comparable protection to that elicited after administration of traditionally attenuated live vaccines (Davison et al., 2006). Several possible reasons have been suggested to explain the inferior level of protection induced following administration of commercial recombinant viral vaccines. These include technical failure to deposit the vaccine in the amniotic cavity or in the embryo muscle during *in ovo* vaccination (Williams and Zedek, 2010) and fractionation of vaccine dose by industry to reduce the cost of vaccination (Dufour-Zavala, 2008; García, 2016). It may also be explained by the inability of virally vectored ILT vaccines to

replicate in respiratory tissues (Coppo et al., 2013b) or the inability of a small number of ILTV proteins included in vectored vaccines to induce immune responses as strong and broad as conventional attenuated vaccines.

1.5.3 Developmental ILTV vaccines

1.5.3.1 Gene-deleted recombinant strains

In response to the increasing needs for more stable, safe and effective ILTV vaccines, several virulence genes have been deleted from the ILTV genome in order to create attenuated recombinant vaccine candidates (Devlin et al., 2006b; Fuchs and Mettenleiter, 2005; García et al., 2016; Helferich et al., 2007; Pavlova et al., 2010; Schnitzlein et al., 1995; Veits et al., 2003a). The suitability of some of these gene-deleted recombinant strains as vaccines is currently being explored. Among the gene-deleted ILTV strains that have shown promising results as a potential vaccine is the glycoprotein G gene-deleted (ΔgG) strain (Devlin et al., 2007). This strain has been assessed as a live attenuated vaccine for eye-drop, drinking water and *in ovo* administration. The level of protection induced by intra-tracheal vaccination with the ΔgG strain was comparable to that induced by the traditionally attenuated SA2 vaccine in SPF chickens (Devlin et al., 2007). Application of the ΔgG strain via drinking water was also shown to be safe and the levels of protection induced were comparable to those achieved

after intra-tracheal vaccination (Devlin et al., 2008). Similarly, Coppo et al. (2011) have shown that the level of protection induced by eye-drop vaccination with the ΔgG strain was comparable to that induced by three traditionally attenuated live vaccines, SA2, A20 and the Serva strain (Coppo et al., 2011).

In ovo vaccination with the ΔgG strain has been shown to be safe and effective in SPF chickens (Legione et al., 2012). Moreover, vaccination with the ΔgG strain has been shown to result in enhanced cell-mediated immune responses, and diminished humoral immune responses (Devlin et al., 2010). As cell-mediated immunity is the principal protective component of immunity against ILTV (Coppo et al., 2018), enhanced cell-mediated immunity after inoculation with the ΔgG strain suggests an additional advantage of the ΔgG strain vaccine. However, the minimum effective dose of this vaccine strain has not yet been determined.

Another gene-deleted recombinant ILTV strain that has shown promise as an attenuated vaccine is the (ORF) C gene-deleted strain (ΔORF C) (García et al., 2016). After eye-drop vaccination the replication of the ΔORF C recombinant strain in the trachea is limited and the level of protection induced was similar to that induced by a TCO vaccine, but less than that elicited by a CEO vaccine. However, the ΔORF C strain was not sufficiently attenuated for *in ovo* delivery (García et al., 2016). Although significant efforts have been made to develop gene-deleted recombinant live vaccines, it is not yet clear whether gene-deleted strains can establish latency in vaccinated birds and/or prevent latent infection

with wild type viruses (Coppo et al., 2013b). The possibility that gene-deleted recombinant strains might revert to virulence during *in vivo* bird-to-bird passage has not yet been established, although the ΔgG ILTV vaccine candidate strain did not increase in virulence following one *in vivo* passage and subsequent infection via contact exposure (Devlin et al., 2011). Although the selective pressure that results in the appearance of more virulent strains may require several *in vivo* passages as shown for traditionally attenuated vaccine strains (Guy et al., 1991), gene-deleted vaccine strains are inherently less likely to revert to virulence because they have a whole gene deleted rather than just a number of point mutations. The possibility that gene deleted ILTV vaccines recombine in the field to generate virulent viruses is yet unclear, suggesting needs to explore this in the future in order to have a product that helps to better control ILT.

1.5.3.2 Alternative viral vector vaccines

Recently, efforts have been made to identify alternative virally vectored vaccines that will be safe, stable and protective against ILTV infection. A naturally occurring low-virulence strain of NDV (LaSota strain) has been used to express immunogenic ILTV glycoproteins (gB, gD, gC) individually or as a multivalent combination and their protective efficacies against both NDV and ILTV have been assessed. Zhao et al. (2014) have constructed two recombinant LaSota NDV vaccine candidates, rLS/ILTV-gB and rLS/ILTV-gD and have assessed their efficacies in both SPF and commercial broiler chickens. Both vaccines elicited

protection against challenge with ILTV at 21 days post-vaccination. Vaccination with rLS/ILTV-gB resulted in more limited tracheal replication of the challenge virus than vaccination with rLS/ILTV-gD. Vaccination of commercial broilers with the rLS/ILTV-gB strain provided protection against disease, but did not limit replication of challenge virus in the trachea as effectively as inoculation with CEO or TCO vaccines (Zhao et al., 2014). In a separate but similar study, three recombinant NDV LaSota strains were constructed that expressed the ILTV glycoproteins gB, gC or gD. The protective efficacy of the recombinants was tested by vaccinating chickens twice with each vaccine, or with combinations of the vaccines. Only the chickens primed and boosted with the NDV strain expressing gD were completely protected against challenge with virulent ILTV. The superiority of this strain was suggested to be attributable to the higher levels of incorporation of gD in the viral envelope and the higher levels of gD expressed on the surface of infected cells than was achieved with the strains expressing gB or gC (Basavarajappa et al., 2014). The prime and boost vaccination strategy used in the latter study may also have contributed to the greater efficacy than was seen in the study conducted by Zhao et al. (2014).

Bacterial artificial chromosomes (BAC) are another method that can be used to develop recombinant live vaccines. A BAC clone of very virulent (vv) Marek's disease virus (MDV isolate Md5 was engineered to delete both copies of the *meq* gene, resulting in complete attenuation of the strain. Glycoprotein B or gJ genes from ILTV were then introduced, creating the BACΔMEQ-gB and BACΔMEQ-gJ recombinant strains, respectively. These viruses have been tested as bivalent

vaccines against MDV and ILTV. The level of protection elicited by the BACΔMEQ-gB recombinant strain was comparable to that induced by a commercial HVT vectored vaccine expressing ILTV gB. However, vaccination with the BACΔMEQ-J recombinant strain did not protect chickens when administered alone, but enhanced protection when administered in combination with BACΔMEQ-gB (Gimeno et al., 2015).

1.5.4 Challenges to the eradication of ILTV

Infectious laryngotracheitis virus had been considered a pathogen that could be eradicated, based on its biological and ecological features. These include the absence of vertical transmission, rapid inactivation of the virus outside the host, the low level of ILTV infectivity, the absence of a wildlife reservoir and the availability of simple industry quarantine procedures that can prevent its spread between sites (Bagust and Johnson, 1995). Despite these features, ILTV has continued to threaten poultry industries globally and some interesting observations about the evolution of ILTV have been described recently (Agnew-Crumpton et al., 2016). This evolution has been facilitated by natural recombination events between distinct ILTV strains that can result in the emergence of virulent viruses (Agnew-Crumpton et al., 2016; Blacker et al., 2011; Lee et al., 2012). Other factors that may add layers of difficulty to ILTV eradication programs include the absence of an ideal or ‘perfect’ vaccine to help control the disease (García, 2016) and the possibility of vaccine viruses ‘spilling over’ into

reservoirs outside the commercial poultry industry, including backyard chickens and even wild birds in some countries (Devlin et al., 2016). Incomplete flock vaccination, which may select for virulent strains as a result of *in vivo* selection pressures, may also challenge eradication programs. Importantly, the absence of appropriate diagnostic tools for monitoring protective flock immune responses after vaccination (Coppo et al., 2013b) has also contributed to difficulties in controlling the disease, as shedding of the virus from unprotected birds within the flock can create a source of infection for other susceptible animals. Moreover, the lack of standardized field vaccination protocols and the endemic nature of the virus in many countries represent significant challenges to ILTV eradication programs.

1.6 ILTV in Australian poultry

Global poultry production is rapidly expanding. Most of the expansion is due to scale-up of intensive farming systems (Devlin et al., 2016). This may trigger the emergence, spread and persistence of pathogens with novel traits (Agnew-Crumpton et al., 2016; Devlin et al., 2016). Infectious laryngotracheitis virus could be a good example of a pathogen that is emerging with novel traits after natural recombination in the field. Disease due to infection with ILTV was first reported in Australia in 1935 (Seddon and Hart, 1935). Since then, Australia has generally harboured a distinct lineage of ILTV, in the absence of the introduction of prevalent international ILTV strains because of the country's strict biosecurity

measures and geographical isolation. Prior to 2006, five different classes (classes 1 - 5) of ILTV had been identified in Australia using PCR-RFLP genotyping techniques (Kirkpatrick et al., 2006). Between 2007 and 2008 an escalation of ILTV outbreaks in Australian poultry resulted in a shortage of two commercially available Australian vaccines, SA2 and A20 (class 1), and a third vaccine, the Serva strain (class 7) was registered and then used in Australia (Blacker et al., 2011). The examination of samples from ILT outbreaks between 2007 and 2009 has uncovered four additional ILTV classes (classes 6 - 9) (Blacker et al., 2011), based on RFLP pattern analysis.

Phylogenetic analysis revealed that class 8 ILTV was genetically related to the Serva vaccine strain (class 7), and it has also been revealed that class 9 ILTV is related to both class 8 and class 7 viruses. Class 8 and 9 viruses were responsible for large outbreaks of disease in New South Wales. It was at first hypothesized that class 8 and 9 viruses may represent subpopulations of virus within the Serva vaccine, which then become dominant strains as a result of *in vivo* selective pressures (Blacker et al., 2011). A seminal study by Lee et al. (2012) that used whole genome sequence analysis found instead that the two new viral genotypes, class 8 and 9, were novel recombinant field strains that emerged as a result of natural recombination events between the Australian vaccines, SA2 and A20 (class 1), and the European vaccine, Serva (class 7) (Lee et al., 2012). The new recombinant class 9 ILTV has an increased level of virulence, replication and transmissibility than the previously dominant strain, class 2 ILTV. These novel traits of the recombinant class 9 ILTV may explain its

improved fitness, and may be related to its dominance in Victoria, where it has now largely displaced class 2 viruses (Lee et al., 2014a).

Further examination of ILTV isolates from outbreaks between 2009 and 2015 has revealed the emergence of another recombinant virus (class 10 ILTV) in New South Wales in 2013. The first case was identified in an unvaccinated 37-day-old broiler flock, which was in close proximity to layer flocks vaccinated with an ILTV vaccine. Subsequently, additional class 10 virus related outbreaks were reported in nearby broiler farms following depopulation of the previously affected broiler flock. Although the parental strains of the recombinant class 10 ILTV are difficult to precisely determine, whole genome sequence analysis strongly indicated that the class 10 virus emerged as a result of recombination events involving the two commercially available ILTV vaccine strains (classes 1 and 7) and a previously detected recombinant, class 8 ILTV. While class 10 was the predominant genotype in New South Wales in 2014, it was not detected in 2015 (Agnew-Crumpton et al., 2016). Comprehensive analyses of natural recombination events in the alphaherpesviruses, including ILTV in Australia, have been described elsewhere (Loncoman et al., 2017).

Although field reports have suggested that class 10 ILTV is highly pathogenic, its virulence, growth rate and transmissibility are yet to be examined under controlled conditions. Interestingly, many of the outbreaks of class 10 ILTV, and also class 9 ILTV, occurred in vaccinated flocks. Administration of an inadequate vaccine

dose, improper vaccine administration or handling, or incomplete flock vaccination may have contributed to the emergence of these strains (Agnew-Crumpton et al., 2016). Taken together, the generation of three novel recombinant ILTVs in Australian poultry industries as a result of recombination events over a short time frame clearly demonstrates the need for improved ILT control strategies and field vaccination programs.

1.7 Research aims

The first aim of the studies described in this thesis was to evaluate the ability of commercially available traditionally attenuated vaccines, and the ΔgG ILTV candidate vaccine, to protect chickens against virulent challenge with the recombinant class 9 ILTV. This is important as class 9 ILTV has become the predominant field strain in Victoria and has resulted in severe outbreaks of disease. The second aim was to determine the minimum effective dose of the ΔgG ILTV vaccine that, when delivered by eye-drop, protects chickens against robust challenge with recombinant class 9 virus. It is crucial to determine the minimum effective dose of ΔgG ILTV as this candidate vaccine strain has consistently shown promise as an alternative attenuated vaccine to control ILTV. The third aim was to develop tools to measure cell mediated immune responses to ILTV to allow the efficacy of vaccination programs to be better assessed. This is important as cell mediated immunity is protective against ILTV, but currently

the capacity to quantify these responses in chickens infected with ILTV is limited because of the lack of suitable T cell assays.

2. Protection induced in broiler chickens following drinking-water delivery of live infectious laryngotracheitis vaccines against subsequent challenge with recombinant field virus

2.1 Introduction

Infectious laryngotracheitis (ILT) is a contagious upper respiratory tract disease of chickens that causes significant economic losses in poultry industries around the world (Bagust, 1986; Bagust et al., 2000; Jones, 2010). The disease is caused by an alphaherpesvirus, infectious laryngotracheitis virus (ILTV), which is classified taxonomically as *Gallid alphaherpesvirus 1 (*Davison, 2010; Adams et al., 2016). In some outbreaks mortality rates of up to 70% have been reported (Fuchs et al., 2007). Attenuated ILTV vaccines have been widely used to control the disease. However, these vaccines have several limitations, including insufficient attenuation (Oldoni et al., 2009), transmission of vaccine virus to unvaccinated birds (Neff et al., 2008; Oldoni et al., 2008), increased virulence after bird-to-bird transmission (Guy et al., 1991) and also incomplete protection in vaccinated birds (Coppo et al., 2012; Fulton et al., 2000).

Recently, two genetically distinct field strains (class 8 and 9 ILTV viruses) were detected in Australia using PCR-RFLP genotyping (Blacker et al., 2011). Evidence from whole genome sequence analysis of the three vaccine strains in use in Australia, along with the genome of these newly emerged strains, confirmed that the class 8 and 9 strains emerged as a result of natural (field) recombination between the recently introduced European-origin vaccine strain (Serva ILTV, MSD Animal Health) and the original Australian vaccine strains (SA-2 and A20 ILTV, Zoeitis) (Lee et al., 2012). The novel recombinant class 9 ILTV strain became the predominant field strain in important poultry producing regions in Victoria, Australia, largely displacing the previously dominant class 2 ILTVs (Blacker et al., 2011) and continues to cause significant outbreaks of disease in commercial poultry flocks (Lee et al., 2012). Recent studies have shown that, compared to class 2 ILTV, class 9 ILTV has enhanced replication kinetics, increased virulence and enhanced potential for horizontal transmission. These differences may help to explain the dominance of class 9 ILTV in the field (Lee et al., 2014a).

Another factor that could contribute to the dominance of class 9 ILTV in the field is the extent to which the virus can be controlled using vaccination. The ability of vaccines to protect birds against challenge with class 9 ILTV has not been investigated previously. This study aimed to examine the extent to which four different live attenuated ILTV vaccines could protect commercial broiler birds against challenge with virulent class 9 ILTV. In order to remain relevant to the

field situations, this study aimed to use conditions similar to those that occur in the field, where possible.

2.2 Materials and methods

2.2.1 Experimental design and viral strains used in this study

Approval for this study (Animal Ethics ID 1312956.1) was granted by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne. One hundred and twenty 10-day-old broilers (obtained from a commercial supplier at 1 day of age) were individually identified with numbered wing-tags and weighed on the day of vaccination (10 days old). Six groups of 20 birds each were placed in separate isolator units and were provided with feed and water *ad libitum*. Three groups were vaccinated with either Serva ILTV, SA-2 ILTV or A20 ILTV via drinking water according to manufacturers' instructions. A fourth group was similarly vaccinated with a glycoprotein G deleted candidate vaccine (ΔgG ILTV) (Devlin et al., 2007) via drinking water at a dose of 10^5 plaque forming units (PFU)/bird. The remaining two groups (negative and positive control groups) were mock vaccinated by addition of sterile cell culture medium to their drinking water. Immediately after vaccination, after the drinkers containing the vaccine had been removed from the isolators, five age-matched unvaccinated birds were added to each group. Twenty days after vaccination, all

birds (including the contact-exposed birds) were inoculated with 10^3 PFU of virulent recombinant class 9 ILTV, except for the birds in the negative control group, which were mock-challenged with sterile cell culture medium. For challenge, half of the viral dose was inoculated into the trachea and half of the dose was administered via eye-drop. The class 9 strain of ILTV had been propagated and titrated as described previously (Lee et al., 2014b).

Four days after challenge, tracheal and conjunctival swabs were collected aseptically using small sterile cotton swab and placed in 1 mL viral transport medium (DMEM, Sigma-Aldrich) supplemented with 10% v/v foetal bovine serum (Sigma-Aldrich), 10 mM HEPES, pH 7.6, 50 µg ampicillin (Sigma-Aldrich)/mL and 2.5 µg amphotericin B (Astral Scientific)/mL). All birds were swabbed to assess viral replication. In addition, five birds that had been vaccinated directly in each group were selected at random and killed by exposure to an overdose of an inhalant anesthetic agent (halothane). These birds were weighed, and proximal tracheal sections were collected and processed for histopathological examination as described previously (Coppo et al., 2011). Seven days after challenge all remaining birds were killed, weighed and samples collected as described above. The severity of viral-induced tracheal lesions, viral detection and replication in the trachea and conjunctiva, and body weight changes were used to assess protection.

2.2.2 Tracheal histopathology

Transverse sections of proximal trachea were collected, processed and stained with haematoxylin and eosin as described previously (Lee et al., 2014a). The severity of the histopathological lesions were scored from 0 (absent) to 4 (severe) as described previously (Guy et al., 1990) by two operators blinded to the group of origin of each of the sections.

2.2.3 Viral detection and quantification

DNA was extracted from 200 µL of tracheal and conjunctival swabs using the QIAxtractor Vx virus kit (Qiagen) and a QIAextractor automated system (Qiagen) as described previously (Lee et al., 2014a). Positive and negative controls were included on each extraction plate. Infectious laryngotracheitis DNA was detected and quantified in the extracted DNA using real-time quantitative PCR and primers that amplify 113 bp of the UL 15 gene of ILTV, as described previously (Mahmoudian et al., 2011). A 10-fold dilution series of the UL 15 sequence cloned into pGEM-T (Promega) was included in duplicate on each plate to enable estimation of the ILTV genome concentration in each of the extracted samples, with the lower limit of detection for the assay defined as 52 genome copies per reaction. Viral genome concentrations were $\log_{10}$ transformed for statistical analysis.

2.2.4 Statistical analysis

Minitab 17 (Minitab Inc, 2010), GraphPad Prism 6 (GraphPad Prism Software) and Excel 2007 (Microsoft) were used to analyse data. Mann-Whitney tests were performed to compare the lesion scores determined by histopathological examination. One-way analyses of variance, in conjunction with Dunnett's Multiple Comparisons tests, were used to compare the viral genome concentrations and percentage body weight gains for the different groups. The normality assumption was assessed using normal probability plots, and equality of variance was checked using Levene's test. Fisher's exact test was used to compare the proportions of ILTV positive birds in each group. A two-tailed $P \leq 0.05$ was considered to be significant.

2.3 Results

2.3.1 Protection in broilers directly vaccinated via drinking-water

Results from assays for viral detection and viral quantification, and tracheal histopathological examination in directly vaccinated birds, four days after

challenge, are summarized in Table 2.1. No significant differences in the severity of tracheal histopathological lesions were detected between groups. Viral genome concentrations were significantly lower in the tracheas of birds vaccinated with Serva, A20, SA-2 or ΔgG ILTV than in the unvaccinated-challenged (positive control) group. In contrast, viral genome concentrations in the conjunctiva did not differ significantly between any of the challenged groups. The proportion of birds in which virus was detected in the conjunctiva and trachea varied between groups. Within the vaccinated groups, the lowest proportions of ILTV positive birds were seen in the group vaccinated with A20 ILTV (for detection of virus in the trachea) and in the group vaccinated with SA-2 ILTV (for detection of virus in the conjunctiva).

Table 2.1 Assays for viral detection and viral quantification, and tracheal histopathological examination in birds directly vaccinated with different ILTV vaccines, four days after challenge

Group		Median tracheal histopathology (range)	Mean log_{10} viral genome copies/reaction ± S. D		Proportion of birds positive for ILTV	
Vaccine	**Challenge**		**Trachea**	**Conjunctiva**	**Trachea**	**Conjunctiva**
None	None	1 (1 - 2) [a]	1.7 ± 0 [a]	1.7 ± 0 [a]	0/17 [a]	0/17 [a]
None	Class 9	2 (1 - 3) [a]	3.4 ± 2.2 [b]	1.8 ± 0.4 [a]	9/20 [b, c]	2/20 [a, c]
Serva	Class 9	2 (1 - 3) [a]	3 ± 1.8 [a]	1.9 ± 0.4 [a]	9/19 [b]	7/19 [b, c]
A20	Class 9	2 (1 - 3) [a]	1.8 ± 0.3 [a]	1.8 ± 0.2 [a]	3/20 [a, c]	5/20 [b, c]
SA-2	Class 9	2 (0 - 3) [a]	2.6 ± 0.9 [a]	1.7 ± 0 [a]	12/18 [b]	0/18 [a]
ΔgG	Class 9	2 (1 - 5) [a]	2.9 ± 2.0 [a]	1.8 ± 0.4 [a]	6/19 [b, c]	1/19 [a]

[a,b,c] Values marked with the same superscripts in the same column were not significantly different (P > 0.05). S. D = standard deviation. The lower limit of detection of the assay used to detect and quantify viral genome was 52, or $10^{1.72}$, genome copies per reaction.

Results from assays for viral detection and viral quantification, and tracheal histopathological examination in directly vaccinated birds in directly vaccinated birds, seven days after challenge, are summarized in Table 2.2. Birds that received the SA-2 ILTV vaccine had significantly less severe upper tracheal histopathology than birds in all other challenged groups. Viral genome concentrations were significantly lower in the tracheas of birds vaccinated with Serva, A20, SA-2 or ΔgG ILTV, than in birds in the unvaccinated-challenged (positive control) group, viral genome concentrations were significantly lower in the conjunctivas of the birds vaccinated with Serva, A20 or SA-2 ILTV, than in those of the birds in the unvaccinated-challenged (positive control group). The proportion of birds in which virus was detected in the conjunctiva and trachea varied between groups. Within the vaccinated groups, the lowest proportions of ILTV positive birds were seen in the groups vaccinated with Serva or A20 ILTV (for detection of virus in the trachea) and in the group vaccinated with SA-2 ILTV (for detection of virus in the conjunctiva).

Table 2.2 Assays for viral detection and viral quantification, and tracheal histopathological examination in birds directly vaccinated with different ILTV vaccines, seven days after challenge

Group		Median tracheal histopathology (range)	Mean log_{10} viral genome copies/reaction ± S. D		Proportion of birds positive for ILTV	
Vaccine	**Challenge**		**Trachea**	**Conjunctiva**	**Trachea**	**Conjunctiva**
None	None	2 (0 - 2) [a]	1.7 ± 0 [a]	1.7 ± 0 [a]	0/12 [a]	0/12 [a]
None	Class 9	3 (0 - 5) [b]	4.6 ± 1.9 [b]	2.9 ± 1.3 [b]	13/14 [b]	12/14 [b]
Serva	Class 9	3 (2 - 5) [b]	2.1 ± 0.9 [a]	1.9 ± 0.5 [a]	4/14 [a, c]	3/14 [a, c]
A20	Class 9	3 (1 - 5) [b]	2.1 ± 0.8 [a]	1.9 ± 0.4 [a]	4/15 [a, c]	3/15 [a, c]
SA-2	Class 9	1 (0 - 4) [a]	2.8 ± 1.3 [a]	1.7 ± 0 [a]	8/13 [b, c]	0/13 [a]
ΔgG	Class 9	3 (0 - 5) [b]	2.8 ± 1.6 [a]	2.8 ± 1.6 [b]	6/15 [c]	8/15 [c, b]

[a, b ,c] Values marked with the same superscripts in the same column were not significantly different (P > 0.05). S. D =standard deviation. The lower limit of detection of the assay used to detect and quantify viral genome concentrations was 52, or $10^{1.72}$, genome copies per reaction.

Percentage weight gains in directly vaccinated birds at three different time points (20 days after vaccination, four days after challenge and seven days after challenge) are summarized in Table 2.3. No significant differences in percentage weight gain were detected between the groups at any of these time points.

Table 2.3 Percentage body weight changes between the day of vaccination and 20 days after vaccination, and between the day of challenge and days four and seven after challenge, in birds directly vaccinated with different ILTV vaccines

Group		Mean percentage body weight change ± S. D					
Vaccine	**Challenge**	**N**	**20 dpv**	**N**	**4 dpc**	**N**	**7 dpc**
None	None	17	381 ± 64 [a]	5	27 ± 6 [a]	12	54 ± 8 [a]
None	Class 9	20	404 ± 91 [a]	5	15 ± 8 [a]	14	48 ± 15 [a]
Serva	Class 9	19	372 ± 98 [a]	5	33 ± 6 [a]	14	56 ± 8 [a]
A20	Class 9	20	414 ± 88 [a]	5	29 ± 12 [a]	15	50 ± 6 [a]
SA-2	Class 9	18	429 ± 84 [a]	5	30 ± 3 [a]	13	48 ± 14 [a]
ΔgG	Class 9	20	380 ± 80 [a]	5	28 ± 14 [a]	15	51 ± 17 [a]

[a] Values marked with the same superscripts in the same column were not significantly different ($P > 0.05$). SD = standard deviation, dpv = days post vaccination, dpc = days post challenge, N = number of birds.

2.3.2 Protection in broilers that were contact-exposed to vaccinated birds

The results from assays for viral detection and quantification four days after challenge in birds that were contact-exposed to vaccinated chickens are summarized in Table 2.4. No significant differences in viral genome concentration, or in the proportion of ILTV positive birds, were seen between groups.

Table 2.4 Assays for viral detection and quantification four days after challenge in birds that were contact-exposed to vaccinated chickens

Group		Mean log_{10} viral genome copies/reaction ± S. D		Proportion of birds positive for ILTV	
Vaccine	**Challenge**	**Trachea**	**Conjunctiva**	**Trachea**	**Conjunctiva**
None	None	1.7 ± 0 [a]	1.7 ± 0 [a]	0/5 [a]	0/5 [a]
None	Class 9	3.3 ± 2.1 [a]	2.0 ± 0.6[a]	3/5 [a]	1/5 [a]
Serva	Class 9	1.7 ± 0 [a]	1.7 ± 0.1[a]	0/4 [a]	1/4 [a]
A20	Class 9	1.7 ± 0 [a]	1.7 ± 0 [a]	0/5 [a]	0/5[a]
SA-2	Class 9	1.9 ± 0.4 [a]	1.7 ± 0 [a]	1/4 [a]	0/4 [a]
ΔgG	Class 9	4.0 ± 2.7 [a]	1.7 ± 0 [a]	2/4 [a]	0/4 [a]

[a] Values marked with the same superscripts in the same column were not significantly different (P > 0.05). S. D =standard deviation. The lower limit of detection of the assay used to detect and quantify viral genome was 52, or $10^{1.72}$, genome copies per reaction.

Results from assays for viral detection and viral quantification, and tracheal histopathological examination seven days after challenge in birds that were contact-exposed to vaccinated birds are summarized in Table 2.5. Birds that were contact-exposed to chickens vaccinated with A20 or ΔgG ILTV had significantly less severe tracheal histopathology scores than birds in the positive control group. Furthermore, birds that were contact exposed to chickens vaccinated with Serva, A20 or SA-2 ILTV had significantly lower ILTV genome concentrations in the trachea than birds in the positive control group. In contrast, no significant reduction in ILTV genome concentrations were seen in the conjunctiva of contact-exposed birds in any of the vaccinated groups compared to birds in the positive control group. The proportions of contact-exposed birds in which virus was detected in the conjunctiva and trachea varied between groups. Within the vaccinated groups, the lowest proportion of ILTV positive contact-exposed birds was in the SA-2 ILTV vaccinated group for detection of virus in the trachea, and in the A20, Serva and ΔgGILTV vaccinated groups for detection of virus in the conjunctiva.

Table 2.5 Assays for viral detection and viral quantification, and tracheal histopathological examinations seven days after challenge in birds that were contact-exposed to vaccinated chickens

Group		Median tracheal histopathology (range)	Mean log_{10} viral genome copies/reaction ± S. D		Proportion of birds positive for ILTV	
Vaccine	**Challenge**		**Trachea**	**Conjunctiva**	**Trachea**	**Conjunctiva**
None	None	1 (0 - 1) [a]	1.7 ± 0 [a]	1.7± 0 [a]	0/5 [a]	0/5 [a]
None	Class 9	4 (2 - 4) [b]	4.7 ± 0.9 [b]	3.1 ± 1.4 [b]	5/5 [b]	5/5 [b]
Serva	Class 9	2 (2 - 4) [b, c]	1.9 ± 0.4 [a]	2.3 ± 1.1 [a, b]	1/4 [a]	1/4 [a]
A20	Class 9	2 (1 - 4) [c]	2.0 ± 0.6 [a]	2.1 ± 0.8 [a, b]	1/5 [a]	1/5 [a]
SA-2	Class 9	3.5 (1 - 5) [b, c]	1.7 ± 0 [a]	2.9 ± 0.8 [b]	0/4 [a]	3/4 [b]
ΔgG	Class 9	2.5 (1 - 4) [c]	4.7 ± 2 [b]	1.9 ± 0.3 [a, b]	3/4 [b]	¼ [a]

[a, b ,c] Values marked with the same superscripts in the same column were not significantly different (P > 0.05). S. D =standard deviation. The lower limit of detection of the assay used to detect and quantify viral genome was 52, or $10^{1.72}$, genome copies per reaction.

Percentage weight gains in birds contact-exposed to vaccinated chickens, at two different time points (20 days after vaccination and seven days after challenge) are summarized in Table 2. 6. The only significant difference was in the group that received the A20 ILTV vaccine. This group had a significantly lower weight gain 20 days after vaccination compared with all other groups.

Table 2.6 Percentage body weight changes between the day of vaccination and 20 days after vaccination, and between the day of challenge and day seven after challenge, in birds that were contact exposed to vaccinated chickens

Group		Mean percentage body weight change ± S. D			
Vaccine	**Challenge**	**N**	**20 dpv**	**N**	**7 dpc**
None	None	5	350 ± 37 [a]	5	53 ± 9 [a]
None	Class 9	5	329 ± 42 [a]	5	49 ± 14 [a]
Serva	Class 9	4	358 ± 57 [a]	4	61 ± 10 [a]
A20	Class 9	5	309 ± 67 [b]	5	51 ± 9 [a]
SA-2	Class 9	4	393 ± 64 [a]	4	47 ± 4 [a]
ΔgGV	Class 9	4	401 ± 142 [a]	4	36 ± 16 [a]

[a,b,] Values marked with the same superscript in the same column at a given time point were not significantly different (P > 0.05). S. D = standard deviation, dpv = days post vaccination, dpc= days post challenge, N= number of birds.

2.4 Discussion

This study aimed to evaluate the ability of attenuated ILTV vaccines to control recombinant, class 9 ILTV under conditions similar to those that occur in the field. Importantly we applied the vaccine via drinking water. Drinking water vaccination is a preferred method for mass delivery of vaccine to large broiler flocks due to the ease and cost effectiveness of application. However, under field conditions, some birds may not consume drinking water containing the vaccine or may receive a suboptimal dose of the vaccine because of reduced consumption. These birds, therefore, remain either unvaccinated or incompletely vaccinated (Coppo et al., 2012; Fulton et al., 2000; Robertson and Egerton, 1981). Other factors may also limit the effectiveness of vaccines delivered via drinking water in poultry, including equipment limitations, poor water quality and sub-optimal preparation and handling of the vaccine (Mutinda et al., 2014). The birds that are not fully vaccinated by drinking water may become infected with vaccine virus following horizontal transmission from vaccinated birds (Devlin et al., 2011), or may remain naïve, creating a small susceptible population within the flock. In order to simulate the lack of uniform vaccination that can occur in commercial flocks vaccinated by drinking water, the present study included five unvaccinated age-matched birds that were placed in-contact with vaccinated birds in each of the groups immediately after vaccination. Their subsequent protection against challenge with virulent virus was assessed.

The study used commercial broiler chickens, instead of specific-pathogen free (layer-type) chickens, in order to ensure its relevance to field situations and commercial broiler industries. Previous studies have demonstrated immunological differences between broiler and layer types of chickens. Broilers produce a strong short-term humoral response, whereas layer-type chickens produce a long-term humoral response in conjunction with a stronger cellular response. These features in broilers appear to be a consequence of genetic selection for economically important traits (Koenen et al., 2002). In this study birds were vaccinated at 10 days of age, consistent with common field vaccination practices in Australia, and challenged at 30 days of age, an age at which ILT outbreaks have commonly been seen in broilers in Australia (Devlin et al., 2011). Age at vaccination and age at challenge are important parameters that can influence protection and disease expression. Previous studies have shown poorer immune responses in chickens vaccinated before two weeks of age due to the immaturity of their cell-mediated immunity, rather than effects of maternally derived antibodies, which are not protective against ILTV (Cover et al., 1960; Gharaibeh and Mahmoud, 2013). However, the short growth cycle in commercial broiler production systems often requires vaccinations to be performed at a younger age than would be immunologically ideal, and so we used a younger age of vaccination in this study.

The results from our study showed that, under conditions resembling field conditions, all the attenuated vaccines induced a level of protection against challenge with recombinant class 9 ILTV in chickens directly vaccinated via drinking water. All

vaccines reduced the amount of detectable virus in the trachea at both four and seven days after challenge compared to unvaccinated birds. All vaccines also reduced the proportion of birds that were positive for the presence of ILTV DNA in conjunctival and/or tracheal swabs seven days after challenge. Furthermore, the SA-2, A20 and Serva ILTV vaccines reduced the amount of virus detected in the conjunctiva seven days after challenge. Reducing the level of viral replication within flocks is important for the control of ILT, as this is likely to reduce the viral load in the environment and potentially decrease the risk of spread beyond the infected flock to new flocks or farms. However, it is important to note that this study only assessed viral load using qPCR detection of viral DNA, which is unable to discriminate between viable and unviable virus (Coppo et al., 2011). Although the qPCR assay did not discriminate between DNA from the challenge virus and DNA from vaccine viruses, past experience with these vaccines in layer birds under similar conditions suggests that vaccine virus is unlikely to have been detectable 24 days after vaccination.

Interestingly, only SA-2 ILTV reduced the severity of tracheal lesions following challenge. Although the SA-2 ILTV strain is known to be highly immunogenic, it is also less attenuated than some other ILTV vaccines, so it is not normally recommended for use in broilers because of concerns about its safety in these birds (Coppo et al., 2011; Devlin et al., 2007; Purcell and Surman, 1974). No significant vaccine safety concerns were noted in this study following SA-2 inoculation, however in the field factors such as stocking rates, housing conditions, and concurrent infection with other pathogens may influence disease expression.

In contact-exposed birds, observations were made and protection deduced against challenge compared to birds that were grouped with unvaccinated chickens. This contrasts with a previous study from the USA (Rodríguez-Avila et al., 2008) in which none of the contact-exposed birds were protected against challenge. The differing outcomes of our study and this previous study could be due to differences in the experimental design, the detection methods used, orthe challenge and vaccine strains used. Contrast to this study, in the mentioned study unvaccinated birds were placed in-contact with vaccinated in 1:1 ratio a day after vaccination, and the period of exposure was for 28 days. They have also, utilised viral isolation and Real-time PCR Taqman assays to detect the virus. In our study, this protection was only seen seven days after challenge and included reduced levels of tracheal pathology (in birds contact-exposed to A20 or ΔgG ILTV vaccinated chickens), reduced concentrations of detectable viral DNA in the trachea (in birds contact-exposed to Serva, A20 or SA-2 ILTV vaccinated chickens) or reduced proportions of birds that had detectable ILTV DNA in conjunctival and/or tracheal swabs (in birds contact-exposed to Serva, A20, SA-2 or ΔgG ILTV vaccinated chickens). This protection was presumably due to horizontal transmission of vaccine virus from directly-vaccinated birds to in-contact birds, although this transmission was not directly assessed. Although transmission of vaccine virus to naïve birds can have potential benefits in terms of inducing a level of protection against challenge, there is also potential for some vaccine strains of virus to revert to higher levels of virulence following bird-to-bird passage (Guy et al., 1991; Oldoni et al., 2008) or the potential for their involvement in recombination events with

other strains of ILTV (Lee et al., 2012). For these reasons field vaccination programs strive to achieve optimal, direct vaccination of all birds to generate uniform protection.

Unexpectedly, no significant difference was seen in percentage weight gain between groups directly vaccinated with the different vaccines in this study. This is in contrast to results from a previous study of class 9 ILTV in which unvaccinated-challenged birds had a significantly decreased weight gain, compared to negative control birds at 6 days after challenge (Lee et al., 2014a). This could be explained by the different environmental conditions that were required for this present study. In particular, light intensity and duration were restricted in order to facilitate the control of aggressive (pecking) behavior seen in some of the chickens. This pecking behavior also necessitated killing of some birds in order to ameliorate suffering, in accordance with animal ethics approval for this work. This resulted in different numbers of birds per group at some time points. In broiler chickens, light restriction programs have been shown to decrease weight gain (Ingram et al., 2000) and so it is possible that these measures to control pecking also restricted weight gain. This may have prevented differences in weight gain between groups being expressed.

In the unvaccinated-challenged birds the assay for viral detection and quantification, and tracheal histopathological examination, revealed some apparent differences in viral replication and disease progression associated with class 9 ILTV compared to another strain of ILTV (CSW-1 ILTV) assessed in previous studies. Following inoculation of class 9 ILTV, the highest viral genome concentrations were seen in the

trachea seven days after challenge. The proportions of ILTV positive birds were also highest seven days after challenge and tracheal lesions were most severe seven days after challenge. In studies utilizing the CSW-1 strain of ILTV (a virulent field virus commonly used as an experimental challenge strain in Australia), tracheal pathology and virus replication reach their highest levels four days after challenge, with most virus cleared from the tracheal mucosa by seven days after challenge (Devlin et al., 2007). The results from this study, in conjunction with previously reported results showing the presence of tracheal pathology and virus replication up to 21 days after infection with class 9 ILTV (Lee et al., 2014a), suggest that the duration of infection and disease is extended for class 9 ILTV. This could be linked to the dominance of class 9 ILTV in the field.

Taken together the results from this study indicate that the currently available attenuated vaccines and the glycoprotein G deleted candidate vaccine can be used to help control class 9 ILTV when delivered by drinking-water. However, it is important to note that neither the protection induced in the directly-vaccinated birds, nor that induced in birds that were contact-exposed to vaccinated birds, was complete. This may help to explain the persistence of class 9 ILTV infection and disease in commercial poultry flocks of Australia, despite the widespread use of vaccination programs similar to those employed in this study. The results highlight the wisdom of combining vaccination programs with other disease control measures, such as biosecurity measures, in order to improve ILT control. Our study also highlights the

need to continue to seek improvements in ILTV vaccines and vaccine delivery methods in order to achieve improved protection against challenge with virulent virus.

3. Determination of the minimum protective dose of a glycoprotein-G-deficient infectious laryngotracheitis virus vaccine delivered via eye-drop to week-old chickens

3.1 Introduction

Infectious laryngotracheitis virus (ILTV) is an alphaherpesvirus that causes acute upper respiratory tract disease in chickens and has significant economic importance for poultry industries throughout the world (Garcia et al., 2013). Commercially available attenuated vaccines are commonly used to help control the disease (Coppo et al., 2013b; García, 2016). Despite the use of these vaccines, many poultry industries continue to experience outbreaks of disease caused by ILTV. In Australia, natural recombination between two distinct live attenuated vaccine strains resulted in the generation of virulent recombinant field strains of ILTV, including the class 8 and 9 viruses (Lee et al., 2012). These recombinant viruses have spread and have caused disease in major poultry-producing areas of Australia. Another recombinant field virus, class 10 ILTV, emerged in 2013 in Australian poultry and has also spread and caused outbreaks of disease (Agnew-Crumpton et al., 2016). Taken together, available data

suggest that improvements to control strategies for ILTV are needed, including tools to control the recently emerged, virulent recombinant viruses.

Recently, efforts to improve the use of ILTV vaccines, and to develop new ILTV vaccines, have resulted in the availability of more options for control of the disease by poultry producers. In some countries, vectored ILTV vaccines are in widespread use. These vaccines use a viral vector, such as fowlpox virus or herpesvirus of turkeys, to deliver specific ILTV antigens (Davison et al., 2006; Esaki et al., 2013; Gimeno et al., 2011; Godoy et al., 2013; Johnson et al., 2010; Vagnozzi et al., 2012). The generation of ILTV mutants deficient in virulence factors (deletion mutant vaccines) has also been investigated and these have the potential to offer additional tools for disease control (Coppo et al., 2013b; García, 2016). Previous *in vivo* and *in vitro* studies have shown that ILTV deficient in the virulence factor glycoprotein G (gG, a chemokine binding protein) has characteristics that would make it suitable for use as an attenuated vaccine (ΔgG ILTV) (Coppo et al., 2018; Coppo et al., 2011; Devlin et al., 2006b; Devlin et al., 2008; Devlin et al., 2007; Devlin et al., 2011; Korsa et al., 2015; Legione et al., 2012; Shil et al., 2012).

The ΔgG ILTV vaccine strain has been extensively studied *in vivo*. The first study to investigate the potential efficacy of the ΔgG ILTV vaccine strain delivered the virus at a dose of 10^3 plaque forming units (PFU), via intratracheal inoculation to four-week-old specific pathogen free (SPF) chickens, followed by challenge of

the birds with the CSW-1 field strain (class 4) of ILTV at seven weeks of age. Birds that received the ΔgG ILTV vaccine had significantly fewer clinical signs than unvaccinated birds (Devlin et al., 2007). The suitability of the ΔgG ILTV vaccine strain was then investigated using delivery methods suitable for mass vaccination programs, including delivery via drinking water, eye-drop or *in ovo* inoculation (Coppo et al., 2011; Devlin et al., 2008; Korsa et al., 2015; Legione et al., 2012). The results from these studies demonstrated that the ΔgG ILTV vaccine strain has desirable safety and efficacy characteristics. Although this vaccine strain has shown promise as a potential alternative to conventionally attenuated live ILTV vaccines, the minimum effective dose required to protect inoculated birds has not been determined. The aim of this study was therefore to determine the minimum dose of ΔgG ILTV that, when delivered by eye-drop to seven-day-old SPF chickens, would protect the birds from a robust challenge with virulent, recombinant class 9 virus.

3.2 Materials and methods

3.2.1 Experimental design, animals and viral strains used in this study

The study was approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne (Animal Ethics

ID -1413097) in accordance with institutional and national guidelines. After hatching, 121 SPF chicks were divided into six groups (five groups with 22 birds and one group with 11 birds) that were each housed in separate isolator units. Vaccine (Vaxsafe ILT) was obtained from the manufacturer (Bioproperties Pty Ltd) as freeze-dried product, five passages beyond the master seed. Inoculum containing the ΔgG ILTV vaccine strain was then prepared by re-suspending the freeze-dried product in commercial sterile diluent (Merial Select Inc.) according to the manufacturer's directions. At seven days of age, four groups, each of which contained 22 birds, were vaccinated with $10^{5.0}$ PFU, $10^{3.8}$ PFU, $10^{3.5}$ PFU, or $10^{3.2}$ PFU of the ΔgG ILTV vaccine strain via eye-drop in a 30 µL volume. The remaining two groups of birds were mock vaccinated by administering the same volume of sterile diluent.

At 28 days of age the vaccinated groups of birds were challenged with class 9 ILTV, a virulent recombinant field strain ($10^{3.0}$ PFU/bird), resuspended in vaccine diluent, with half of the dose delivered onto the conjunctiva (40 µL each eye) and the remaining half into the trachea (150 µL total volume). Similar procedures were used to inoculate one of the unvaccinated groups of 22 birds with the same virulent field strain, as a positive control group (unvaccinated-challenged). The remaining unvaccinated group of 11 birds was kept as a negative control group (unvaccinated-unchallenged). Birds were scored for clinical signs of disease from three to six days post-challenge (dpc). Typical clinical signs due to challenge with wild type virus are expected to peak during this period (Devlin et al., 2008). Any birds that showed severe signs of disease were killed by anaesthetic (halothane)

overdose. All remaining birds were killed at 7 dpc. At necropsy, the severity of ILTV induced tracheal pathology was recorded (Devlin et al., 2008), and conjunctival and tracheal swabs were collected aseptically using a small sterile pre-moistened cotton swab. Each swab was immediately placed in 1 mL of sterile viral transport medium consisting of Dulbecco's minimal essential medium (DMEM, Sigma-Aldrich) supplemented with 10% v/v foetal bovine serum (Sigma-Aldrich), 10 mM HEPES, pH 7.6, 50 µg ampicillin (Sigma-Aldrich)/ml and 2.5 µg amphotericin B (Astral Scientific)/ml to quantify viral load in these sites. The samples were transported on ice and then immediately stored at -80°C until processing. Clinical scores, gross pathological lesions, viral detection and replication in the conjunctival and tracheal mucosa were used to assess the level of protection induced in response to the different doses of ΔgG ILTV and are described in further detail below.

3.2.2 Clinical scoring

Clinical signs of demeanour, dyspnoea, and conjunctivitis were scored as previously described (Devlin et al., 2007). Briefly, demeanour was scored as 0 (normal demeanour), 1 (depressed demeanour) or 2 (severely depressed demeanour). Similarly, conjunctivitis was scored as 0 (conjunctival mucosa normal), 1 (partial eye closure) or 2 (compete eye closure, marked conjunctivitis), and dyspnoea was scored as 0 (normal breathing), 1 (mild dyspnoea), 2 (moderate dyspnoea), 3 (marked dyspnoea), or 4 (severe gasping).

3.2.3 Gross tracheal pathology scoring

Gross tracheal pathology was scored as previously described (Devlin et al., 2007). Briefly, at necropsy, the trachea was removed and then cut lengthways for gross lesion scoring. The severity of the lesions was scored as 0 (normal),1 (mild amount of mucus present), 2 (moderate amount of mucous present), 3 (large amount of mucus present, some blood also present, or diphtheritic material present but not appearing to block the trachea) or 4 (large amount of mucus present, significant blood also presents, or a diphtheritic plug present and blocking the trachea).

3.2.4 Viral detection and quantification

DNA was extracted from the medium containing the conjunctival and tracheal swabs that were collected during necropsy using the KingFisher Flex Purification System (ThermoFisher Scientific) following the manufacturer's instructions. Positive extraction control samples (diluted stocks of the SA2 ILTV vaccine strain; Zoetis) and negative extraction control samples (distilled water) were included in each extraction plate. Ninety microliter samples of eluted DNA were sealed and stored at -20°C until the extracts were tested for the presence of ILTV DNA by real-time quantitative PCR using oligonucleotide primer pairs that amplify a 113

bp region of the UL 15 gene of ILTV, as described previously (Mahmoudian et al., 2011). To generate a standard curve, a 10-fold dilution series of the UL15 sequence cloned into pGEM-T (Promega) was prepared using a QIAgility robot (Qiagen) and included in triplicate in each run to enable quantitation of the ILTV genome concentration in each of the extracted samples. Only samples that produced amplicons with a melt curve that matched those of the standard curve samples were regarded as positive for the presence of ILTV DNA. Calculations of the concentration of the ILTV genome in the extracted samples were performed using Rotorgene Q version 2.1.0 (Qiagen) and the concentration in viral genome copy numbers per reaction were log10 transformed for statistical analysis, with the lower limit of detection for the assay defined as 100 genome copies per reaction.

3.2.5 Statistical analyses

GraphPad Prism 6 (GraphPad Software) and Excel 2016 (Microsoft) were used for data analyses. Mann-Whitney tests were used to compare differences between the groups in the scores for clinical signs and gross tracheal pathology. A one-way analysis of variance, in conjunction with Dunnett's Multiple Comparisons test, was used to compare differences between the groups in viral genome concentrations. Fisher's Exact Test was used to compare differences between the groups in the proportions of birds positive for ILTV by qPCR and in mortality rates. A two-tailed $P \leq 0.05$ was considered to be significant.

3.3 Results

Mortality rates, the severity of viral-induced clinical signs of disease (demeanour, conjunctivitis and dyspnoea), the severity of gross tracheal pathology, and the level of viral replication in tracheal and conjunctival mucosa were assessed after challenge. The vaccinated and control groups were compared in order to identify a suitable minimum effective dose of the vaccine.

3.3.1 Clinical signs of disease

Scores for demeanour, conjunctivitis and dyspnoea are summarised in Tables 3.1, 3.2 and 3.3, respectively. For all these parameters, the highest scores were seen in the positive control (unvaccinated-challenged) group, and the scores in the negative control (unvaccinated-unchallenged) group were all zero. The differences between the negative control and the positive control group were significant for all parameters. There were no significant differences between the group that received the highest dose of vaccine ($10^{5.0}$ PFU) and the negative control group at any time point for any of these parameters. This was also true for the group that received the second highest dose of vaccine ($10^{3.8}$ PFU), although when the scores were summed to yield cumulative scores, this group

had a cumulative conjunctivitis score that was significantly higher than the cumulative score for the negative control group (Table 2). The scores for the groups of birds that received the lower doses of vaccine ($10^{3.2}$ or $10^{3.5}$ PFU) were significantly higher than those for the negative control group for all disease parameters on at least two of the four time points. In these groups, the cumulative scores for all parameters were significantly higher than those of the negative control group (Tables 3.1 – 3.3).

Table 3.1 Scores for demeanour on days 3 to 6 after challenge in unvaccinated birds and in birds vaccinated with different doses of the ∆gG ILTV vaccine administered via eye-drop

∆gG ILTV vaccine dose (PFU)	Challenge	Median demeanour score (range)*				
		3 dpc^	4 dpc	5 dpc	6 dpc	Cumulative†
None	None	0 (0 - 0) [A]	0 (0 - 0) [A]	0 (0 - 0) [A]	0 (0 - 0) [A]	0 (0 - 0) [A]
None	Class 9‡	1 (0 - 2) [B]	1 (1 – 2) [B]	1 (1 - 2) [B]	1 (1 - 1) [B]	3 (0 - 5) [B]
$10^{5.0}$	Class 9	0 (0 - 0) [A]	0 (0 - 0) [A]	0 (0 - 0) [A]	0 (0 - 0) [A]	0 (0 - 0) [A]
$10^{3.8}$	Class 9	0 (0 - 1) [A]	0 (0 - 1) [A]	0 (0 - 1) [A]	0 (0 - 1) [A]	0 (0 - 3) [A]
$10^{3.5}$	Class 9	0 (0 - 1) [A]	0 (0 - 2) [C]	1 (0 - 2) [C]	0 (0 - 1) [A]	1 (0 - 5) [C]
$10^{3.2}$	Class 9	0 (0 - 1) [A]	0 (0 – 1) [A, C]	1 (0 - 2) [C]	0 (0 - 1) [A]	1 (0 - 4) [C]

*Values labelled with the same uppercase superscript letter in the same column were not significantly different (P > 0.05, Mann-Whitney test)

‡ Class 9 challenge = 10^3 PFU of class 9 ILTV delivered via eye-drop and intra-tracheal inoculation

^ dpc = days post-challenge

† Cumulative = sum of scores on all days for each individual bird.

Table 3.2 Scores for conjunctivitis on days 3 to 6 after challenge in unvaccinated birds and in birds vaccinated with different doses of ΔgG ILTV vaccine administered via eye-drop

ΔgG ILTV vaccine dose (PFU)	Challenge	Median conjunctivitis score (range)*				
		3 dpc^	4 dpc	5 dpc	6 dpc	Cumulative†
None	None	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}
None	Class 9‡	0 (0 - 1) ^{B}	1 (0 - 2) ^{B}	1 (0 - 2) ^{B}	1 (0 - 1) ^{B}	1 (0 - 6) ^{B}
$10^{5.0}$	Class 9	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) $^{A, C}$
$10^{3.8}$	Class 9	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 2) ^{A}	0 (0 - 0) ^{A}	0 (0 - 2) ^{C}
$10^{3.5}$	Class 9	0 (0 - 1) ^{A}	0 (0 - 1) $^{A, C}$	0 (0 - 2) ^{A}	0 (0 - 2) ^{C}	0 (0 - 5) ^{D}
$10^{3.2}$	Class 9	0 (0 - 1) ^{B}	0 (0 - 2) ^{C}	0 (0 - 1) ^{A}	0 (0 - 1) $^{B, C}$	0 (0 - 4) ^{D}

*Values labelled with the same uppercase superscript letter in the same column were not significantly different (P > 0.05, Mann-Whitney test)

‡ Class 9 challenge = 10^3 PFU of class 9 ILTV delivered via eye-drop and intra-tracheal inoculation

^ dpc = days post-challenge

† Cumulative = sum of scores on all days for each individual bird.

Table 3.3 Scores for dyspnoea on days 3 to 6 after challenge in unvaccinated birds and in birds vaccinated with different doses of ΔgG ILTV vaccine administered via eye-drop

ΔgG ILTV vaccine dose (PFU)	Challenge	Median dyspnoea score (range)*				
		3 dpc^	4 dpc	5 dpc	6 dpc	Cumulative†
None	None	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}
None	Class 9‡	1 (0 - 3) ^{B}	1 (0 - 3) ^{B}	1 (0 - 2) ^{B}	0 (0 - 1) ^{B}	2 (1 - 6) ^{B}
$10^{5.0}$	Class 9	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}
$10^{3.8}$	Class 9	0 (0 - 1) ^{A}	0 (0 - 1) ^{A}	0 (0 - 2) ^{A}	0 (0 - 1) ^{A}	0 (0 - 4) $^{A, C}$
$10^{3.5}$	Class 9	0 (0 - 1) ^{A}	0 (0 - 2) ^{A}	0 (0 - 3) ^{B}	0 (0 - 1) ^{B}	1 (0 - 4) ^{C}
$10^{3.2}$	Class 9	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}	0 (0 - 0) ^{A}

*Values labelled with the same uppercase superscript letter in the same column were not significantly different (P > 0.05, Mann Whitney test)

‡ Class 9 challenge = 10^3 PFU of class 9 ILTV delivered via eye-drop and intra-tracheal inoculation

^ dpc = days post-challenge

† Cumulative = sum of scores on all days for each individual bird.

3.3.2 Viral detection and quantification in tracheal and conjunctival swabs

The results for viral quantification in tracheal and conjunctival swabs after challenge are presented in Figure 3.1. The groups vaccinated with the two higher doses of the vaccine ($10^{3.8}$ PFU or $10^{5.0}$ PFU) had concentrations of virus at both sites that were significantly lower than those of birds in the positive control group, but that were not significantly different from those of the negative control (unvaccinated-unchallenged) birds. The groups vaccinated with the two lower doses of vaccine had concentrations of virus at both sites that were significantly higher than those of negative control birds. There was no significant difference in viral concentrations in the trachea of the birds that received the two lower doses of vaccine and that of birds in the positive control group, but the two lower doses of the vaccine were partially protective in the conjunctiva, resulting in significantly lower concentrations of virus at this site than in the positive control birds (Figure 3.1).

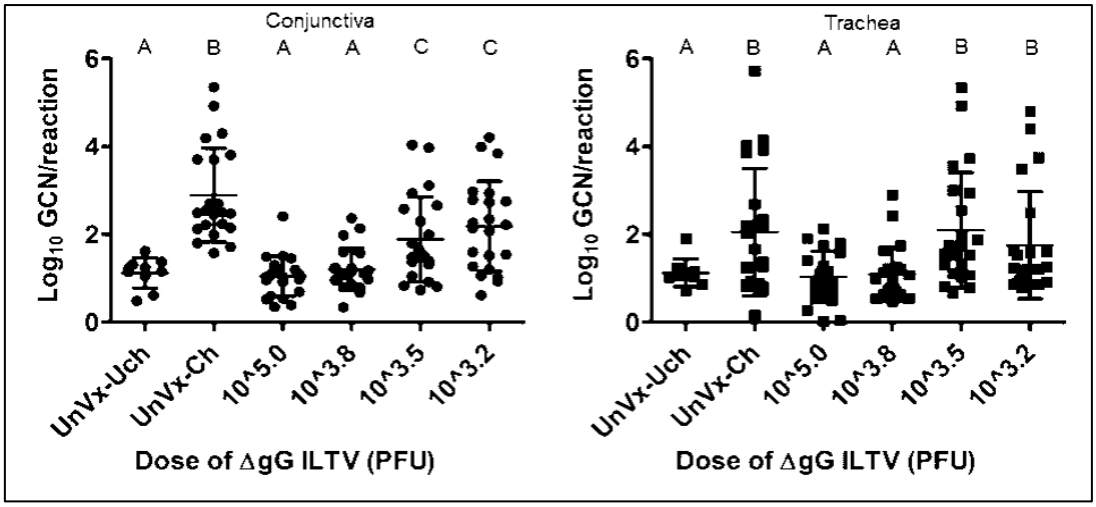

Figure 3.1 Scatter plot of concentrations of ILTV determined by qPCR in swabs. Extracts from conjunctival or tracheal swabs of unvaccinated birds and birds vaccinated with different doses of ∆gG ILTV at day 7 after challenge with class 9 ILTV were subjected to qPCR. Values labelled with the same upper-case letter (A, B, C) were not significantly different (P > 0.05, one-way analyses of variance in conjunction with Dunnett's Multiple Comparisons test) in each panel (conjunctiva or trachea). GCN = genome copy number, UnVx-Uch = unvaccinated and unchallenged, UnVx-Ch = unvaccinated and challenged, PFU = plaque forming units. Means and standard deviations are also shown. The cut off value for positivity was 100 genome copies per reaction.

The proportions of birds that were positive after challenge for ILTV by qPCR are shown in Figure 3.2. A smaller proportion of birds that received the higher doses of the vaccine ($10^{3.8}$ PFU or $10^{5.0}$ PFU) yielded ILTV positive conjunctival swabs (1/22 and 2/22 birds, respectively) and these proportions did not differ

significantly different from that of the negative control group (0/22 ILTV positive birds). In the group that received the lowest dose of vaccine, the proportion of ILTV positive birds (13/22) did not differ significantly different from that of the positive control group (18/22). Fewer significant differences were seen between groups in the proportions of birds positive for ILTV DNA in tracheal swabs (Figure 3.2).

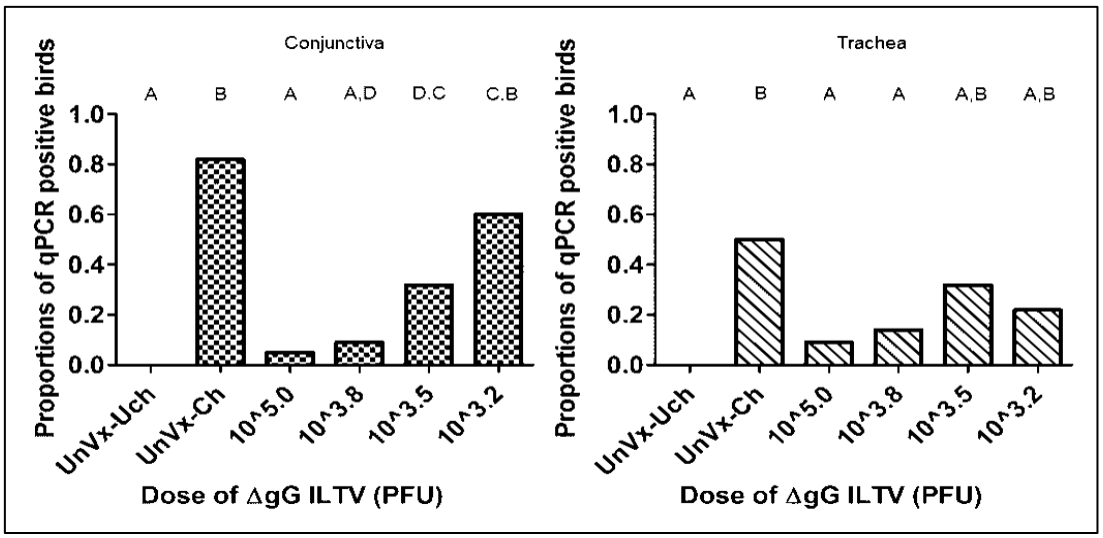

Figure 3.2 Bar graphs showing proportions of qPCR ILTV positive birds. Extracts from conjunctival or tracheal swabs of unvaccinated birds and birds vaccinated with different doses of ΔgG ILTV at day 7 after challenge with class 9 ILTV were tested. Positivity was determined using the cut-off value of 100 genome copies number per reaction. UnVx-Uch = unvaccinated and unchallenged, UnVx-Ch = unvaccinated and challenged, PFU = plaque forming units. Values labelled with the same upper-case letter (A, B, C, D) were not significantly different (P > 0.05, Fisher's exact test) in each panel (conjunctiva or trachea).

3.3.3 Gross tracheal pathology and mortalities

Mortalities and gross tracheal pathology scores after challenge are shown in Table 3.4. No mortalities were seen in the birds vaccinated with the higher doses of vaccine ($10^{3.8}$ PFU or $10^{5.0}$ PFU). There were four mortalities after challenge in each of the groups of birds that received the lower doses of vaccine ($10^{3.2}$ PFU or $10^{3.5}$ PFU). These mortality rates were not significantly different from that seen in the positive control group (10/22).

Table 3.4 Mortality rates and gross pathological scores in unvaccinated birds and in birds vaccinated with different doses of ∆gG ILTV vaccine via eye-drop at day 7 after challenge

∆gG ILTV vaccine dose (PFU)	Challenge	N*	No. mortalities‡^ (%)	Median pathology (range)
None	None	10	0 (0) [A, C]	0 (0 - 0) [A]
None	Class 9‡	22	10 (45.5) [B]	1.5 (0 - 3) [B]
$10^{5.0}$	Class 9	22	0 (0) [A]	0 (0 - 1) [A]
$10^{3.8}$	Class 9	22	0 (0) [A]	0 (0 - 1) [A]
$10^{3.5}$	Class 9	22	4 (18.2) [B, C]	1 (0 - 3) [C]
$10^{3.2}$	Class 9	22	4 (18.2) [B, C]	1 (0 - 3) [C]

* N = number of birds in group

‡ Values labelled with the same uppercase superscript letter in the same column were not significantly different (P > 0.05, Fishers exact test or Mann-Whitney test)

‡ Class 9 challenge = 10^3 PFU of class 9 ILTV delivered via eye-drop and intra-tracheal inoculation

^ Birds that died or were killed due to severe clinical signs of disease following challenge.

None of the birds in the negative control group had any tracheal pathology consistent with infection with ILTV. There was no significant difference in the tracheal pathology scores of the birds in the negative control group and those of the birds in the groups that received the two higher doses of vaccine ($10^{3.8}$ PFU

or $10^{5.0}$ PFU). The tracheal pathology scores of the groups of birds that received the lower doses of vaccine ($10^{3.2}$ PFU or $10^{3.5}$ PFU) were significantly higher than the scores of the birds in the negative control group, but significantly lower than the scores of the birds in the positive control group.

3.4 Discussion

A dose of $10^{3.8}$ PFU was the lowest dose capable of providing a high level of protection against challenge with the class 9 ILTV. In this study, birds vaccinated with a dose of $10^{3.8}$ PFU were protected and did not develop clinical signs or tracheal pathology consistent with ILT. Replication of wild type virus was also greatly reduced in the conjunctival and tracheal mucosa of these birds but was not completely prevented. This is common for ILTV vaccines, which do not typically induce sterilising immunity (Bagust and Johnson, 1995; Coppo et al., 2011; Devlin et al., 2008; Guy et al., 1990; Korsa et al., 2015). Increasing the vaccine dose to $10^{5.0}$ PFU did not significantly improve the level of protection compared to that induced by the minimum effective dose, whereas lower doses ($10^{3.2}$ PFU or $10^{3.5}$ PFU) failed to fully protect the birds from clinical signs after challenge. Unprotected or incompletely protected birds may serve as reservoirs of infection from which susceptible animals may become infected (Agnew-Crumpton et al., 2016). Thus, vaccines should not be used at a dose below the minimum effective dose.

Vaccination and challenge experiments are commonly performed to assess the level of protection induced by ILTV vaccines. Such studies are necessary because there are currently no easily measured correlates of protection (such as serum antibody levels) for ILTV infection, as cell mediated immunity (Fahey et al., 1984; Honda et al., 1994a), rather than neutralising antibodies (Fahey and York, 1990), are thought to protect against disease. Most ILTV vaccination and challenge studies assess levels of clinical protection by scoring clinical signs, tracheal pathology, and mortalities (Coppo et al., 2013b; Devlin et al., 2008; García, 2016; Rodríguez-Avila et al., 2008). Many also assess weight gain (Coppo et al., 2011; Korsa et al., 2015; Rodríguez-Avila et al., 2008). Weight gain was not measured in this study as it used layer type birds, which do not typically have significant weight gains in such short period. Importantly, our study also assessed viral replication after challenge by measuring the concentrations of viral genomes, as have some other vaccine efficacy studies (Coppo et al., 2011; Devlin et al., 2008; Johnson et al., 2010; Korsa et al., 2015). Vaccines that prevent clinical signs of disease but do not prevent replication of pathogens after challenge have been called ‘leaky’ or ‘imperfect’ vaccines, and may select for more virulent pathogens over time (Devlin et al., 2016; Read et al., 2015), as has recently been demonstrated experimentally for Marek’s disease vaccines (Read et al., 2015). In our study, the concentrations of viral genomes in the conjunctival and tracheal mucosa were reduced to very low levels in birds that received high doses of the vaccine ($10^{3.8}$ PFU or $10^{5.0}$ PFU) and only a small number of birds had detectable concentrations of ILTV DNA in the trachea or conjunctiva. These results suggest this vaccine induces significant protection against viral replication, although it should be noted that viral detection and quantification was

assessed at one time point. The inclusion of additional time points would further clarify this observation.

The results from this study demonstrate that Vaxsafe ILT induces a high level of protection when administered by eye-drop at a dose of $10^{3.8}$ PFU. This adds to the growing number of studies that have demonstrated the high level of safety and efficacy of the ΔgG ILTV strain when administered via different routes, using different challenge models (Coppo et al., 2011; Devlin et al., 2008; Devlin et al., 2007; Legione et al., 2012). Glycoprotein G (gG) is a viral chemokine binding protein. Deletion of gG from the ILTV genome alters the immune response to ILTV infection (Devlin et al., 2010), resulting in a cell-mediated immune response, which is more protective than humoral immunity (Coppo et al., 2018). This vaccine also offers the potential to discriminate between vaccinated birds and birds infected with wildtype viruses using differential PCR (Shil et al., 2015) or ELISAs (Shil et al., 2012) in DIVA (differentiation of infected and vaccinated animals) control programs. Although a number of gene-deleted ILTV strains have been shown to have *in vivo* phenotypes that may make them suitable for use as vaccines (Coppo et al., 2011; Devlin et al., 2006b; Devlin et al., 2008; Devlin et al., 2007; Fuchs and Mettenleiter, 2005; García et al., 2016; Han et al., 2002; Helferich et al., 2007; Legione et al., 2012; Mashchenko et al., 2013; Pavlova et al., 2010; Schnitzlein et al., 1995; Veits et al., 2003a), the ΔgG ILTV vaccine strain is the most extensively investigated of these deletion mutants, providing a high level of confidence in the performance of the vaccine under different conditions. This current study shows that the ΔgG ILTV vaccine strain is able to induce a high

level of protection against a virulent recombinant field virus at a commercially feasible dose, and these results lay the foundations for the application of a commercial vaccine product, offering poultry producers a new tool to help control ILTV

4. Development of tools to measure infectious laryngotracheitis virus specific chicken interferon gamma production in an attempt to measure cell mediated immunity post vaccination

4.1 Introduction

Infectious laryngotracheitis virus is an alphaherpesvirus that causes an acute upper respiratory tract disease in chickens and causes significant economic loses to the poultry industry worldwide (Garcia et al., 2013). The virus is horizontally transmitted from infected to susceptible birds resulting in large outbreaks of disease (Devlin et al., 2011). Conventionally attenuated commercial vaccines are commonly used in layer poultry production systems to control the disease (Coppo et al., 2013b; García, 2016), while in the meat chicken industry, vaccination may not be routinely used unless disease outbreaks occur (Bagust et al., 2000). Currently available commercial attenuated vaccines are widely used but have limitations including establishment of latency in vaccinated birds, transmission from vaccinated to unvaccinated birds, recombination between different strains to generate virulent field strains and reversion of virulence following bird-to-bird passage (Agnew-Crumpton et al., 2016; Bagust, 1986; Coppo et al., 2012; Fuchs

et al., 2007; Guy et al., 1991; Lee et al., 2012; Oldoni et al., 2008; Rodríguez-Avila et al., 2007, 2008; Williams et al., 1992).

Currently the success of ILTV vaccination programs is assessed by detecting ILTV neutralizing antibodies in blood post vaccination using ELISA. However, since the presence or titre of antibody does not correlate with the level of protection, the antibody status is unable to properly assess vaccine induced protection (Coppo et al., 2013b; Sander and Thayer, 1997; Shil et al., 2012). Cell mediated responses have been investigated using immuno-histochemical staining, where Devlin et al. (2010) have demonstrated an increased number of $CD8^+$ and $CD4^+$ T lymphocytes in the tracheal sections of birds inoculated with a glycoprotein G deficient ILTV vaccine candidate (Devlin et al., 2010). A range of different possible assay formats exists to assess CMI responses including lymphoproliferation assays (Chen et al., 2011; Smith et al., 2001). However, these are time consuming and can be technically challenging to adapt to a high throughput format. Furthermore, the lack of correlation between proliferative responses and quantification of CMI activity when assessed using IFN-γ production by the same lymphocyte cultures using IFN-γ specific ELISA (Lambrecht et al., 2004) and poor sensitivity of detection also challenges their utility in this context. Development of better tools to quantify CMI responses to ILTV vaccinations may help discriminate protected from unprotected birds. Therefore, the aims of this study were to develop chicken interferon gamma ELISA (ChIFGAM ELISA) and ILTV-specific splenocyte stimulation assays and

to assess their utility as suitable tools to measure chicken CMI responses to ILTV vaccinations.

4.2 Materials and methods

4.2.1 Viral culture and ILTV antigen preparation

Infectious laryngotracheitis virus (class 9) was propagated using male leghorn chicken hepatoma (LMH) cell lines. LMH cells were cultured as described previously (Devlin et al., 2006a). The class 9 isolate was first described by (Blacker et al., 2011), sequenced by Lee et al. (2012) and propagated as described previously (Lee et al., 2014a).

Preparations of semi-purified ILTV virions were made for use in the splenocyte stimulation assay. Briefly, ILTV infected LMH monolayers (5950 cm^2 equivalent) were subjected to a freeze-thaw cycle before culture supernatants were clarified by centrifugation at 524 *x g* for 15 min and then 2,851 *x g* for another 15 min at 4°C. Virus was then pelleted from the clarified supernatants by centrifugation at 52,112 *x g* at 4°C for 1 h and the resultant pellet was resuspended in 4 mL PBS (pH 7.4) before layering onto 4 mL of 5% w/v sucrose in PBS and centrifugation at 112,700 *x g* at 4°C for 1 h. Supernatant was discarded, and the pellet was

resuspended into 2 mL sterile PBS and divided into 100 µL aliquots for storage at -80°C. The same procedures, with exception of ILTV infection, were followed to generate negative control LMH antigens.

To UV inactivate semi-purified viral preparations, 300 µL of ILTV and LMH suspensions were placed in separate wells of a 6-well culture tray (Costar). The tray was kept (lid off) on ice 30 cm away from the UV light inside a class II biosafety cabinet. The UV light was switched on and the suspensions were incubated for 45 min. To determine the level of inactivation, compared to untreated controls, both the untreated virus and the UV treated virus were titrated using plaque assay as previously described (Devlin et al 2006). The semi-purified ILTV antigen and the UV treated antigen had titres of 1.4×10^8 and 10 PFU/mL respectively, which is a 7.15 $\log_{10}$ reduction in titre after UV treatment.

4.2.2 ChIFGAM ELISA assay

Wells of a 96-well plate (Nunc, Thermo scientific) were coated overnight at 4°C with 50 µL of 3 µg/mL of rabbit anti-chicken IFN-γ polyclonal antibody (Kingfisher Biotech) in 0.1 M carbonate/0.1 M bicarbonate coating buffer pH 9.6. Following two washes in PBST (PBS with 0.05% v/v Tween 20), a blocking solution (1% w/v bovine serum albumin (BSA) fraction V, Roche) in PBS with 0.1% v/v Tween 20) was added and then incubated for 1 h at room temperature with orbital

shaking. The plate was then washed twice with 400 µL/well of PBST. Supernatants harvested from stimulated splenocyte cultures (test samples) or dilutions of recombinant chicken IFN-γ protein (Kingfisher Biotech, cat) standard curves diluted in ELISA diluent (5 mg/mL BSA in PBST) or ELISA diluent only (negative control) were then added in triplicate (50 µL per well) and the plate was incubated at room temperature for 2 h with orbital shaking. The plate was washed four times with PBST at this and all subsequent wash steps, before the addition of 50 µL/well of 3 µg/µL biotinylated rabbit anti-chicken recombinant IFN-γ polyclonal antibody (Kingfisher Biotech) was added at 3 µg/mL (final concentration) in ELISA diluent. After 2 h incubation at room temperature with continual shaking, the plate was washed and horseradish peroxidase conjugated streptavidin (DAKO), was added at 1/1000 dilution (50 µL per well) and then further incubated at room temperature for another 1 h while shaking. After the final wash step, the assay was developed by adding 50 µL per well of soluble 3, 3', 5, 5'-tetramethylbenzidine chromogen (Sigma) according to manufacturer's instructions. After incubation at room temperature for 20-30 min the reaction was stopped by the addition of 50 µL 1 M HCl. Absorbance at 450 nm was read in a Synergy HI Hybrid reader (BioTek) using the Gen 5 software (BioTek). The amount of IFN-γ detected in supernatants was quantified by interpolating from a standard curve generated using commercial recombinant chicken IFN-γ included in each ELISA run.

4.2.3 Splenocyte stimulation assay

To optimise conditions for splenocyte stimulation, spleen tissues were obtained from SPF chickens approved for a separate study (Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Approval 1513713.1). During post-mortem examination, spleen tissues were collected into splenocyte culture medium (DMEM) supplemented with 10% v/v FBS, 50 µg/mL ampicillin, 50 µg/mL co-trimoxazole, 50 µg/mL amphotericin B, 50 mM β-mercaptoethanol (β-ME) and 2 mM glutamine). The samples were transported on ice and immediately processed. To prepare single splenocyte suspensions, the capsule was removed from the spleen prior to passage through 100 µm cell strainer (Corning) using gentle pressure applied with a 10 mL syringe plunger. Splenocytes were isolated by density gradient centrifugation for 30 min at 400 *x g* using FICOLL Paque PLUS and washed twice in 12 mL of culture media (1,000 *x g* 10 min, 4°C). Finally, cells were resuspended in 1 mL culture medium and viable cells were quantified by trypan blue dye exclusion in a hemocytometer. For *in vitro* stimulation assays, the final volume of cells and mitogen/antigen was 200 µL per well, with splenocytes plated into 96-well flat-bottomed plate (Sigma-Aldrich) in triplicate at 2 x 10^6 cells/well in splenocyte culture media. Assays included splenocytes incubated with medium alone (unstimulated control and concanavalin A (Con A, Sigma) at 40 µg/mL as the positive control. Other assays examined stimulation by a combination of 100 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma) with 500 ng/mL ionomycin (I, Sigma) or a semi-purified ILTV (specific antigen) or LMH (negative control

antigen) was used at 1/10 final dilution. After 48 h of incubation at 41°C in a humidified atmosphere of 5% v/v CO_2, culture supernatant was harvested and stored at -20°C until tested for IFN-γ using ChIFGAM ELISA.

4.2.4 In vivo infection study

Specific pathogen free (SPF) chickens used in these studies were hatched from eggs provided by Australian SPF Services Pty Ltd (Australia) and were kept in isolators with provision of irradiated food and sterile water ad libitum. The *in vivo* infection study was approved by the Animal Ethics Committee of the Faculty of Veterinary and Agricultural Sciences, The University of Melbourne (Animal Ethics ID-1414129) in accordance with institutional and national guidelines. At 3 weeks of age, 25 SPF chickens were allocated into three groups (two groups of 10 and one group of 5 birds). Birds in the first and second groups containing 10 birds were inoculated with 10^3 PFU/bird of either class 9 ILTV or SA2 ILTV (a vaccine strain), where half of the dose was administered into the trachea (500 PFU/300 µL) and half of the dose administered into the conjunctiva by eye-drop (500 PFU/30 µL). The third group containing 5 birds was mock inoculated with sterile culture medium. Thirty-five days post challenge, all birds were killed using halothane, and spleen tissues were collected during necropsy and processed immediately as described above.

4.2.5 Statistical analysis

GraphPad Prism 6 (GraphPad Prism Software) and Excel 2016 (Microsoft) were used to analyse the data. A Student's t-test was used to compare the level of IFN-γ production between different treatment groups. The level of significance was set at $P < 0.05$.

4.3 Results

4.3.1 ChIFGAM ELISA

Concentrations of detection and capture polyclonal antibodies to chicken IFN-γ were titrated to optimise detection of recombinant IFN-γ protein (positive control) compared to a negative control (Figure 4.1). The optimal concentration of capture and detection antibody was determined at 3 µg/mL for both antibodies. The analytical sensitivity of the assay was 36 pg/mL recombinant IFN-γ protein (Figure 4.2).

To test the capacity of ChIFGAM ELISA to detect IFN-γ produced in splenocyte cultures, chicken splenocytes were stimulated either with Con A or PMA + I

mitogens or medium alone (Figure 4.3). The highest concentration of IFN-γ was detected in culture supernatants obtained from Con A stimulated splenocytes compared to those obtained from unstimulated splenocytes or those stimulated with a combination of PMA+ I. Therefore, Con A was selected to be used as positive control for subsequent stimulation assays.

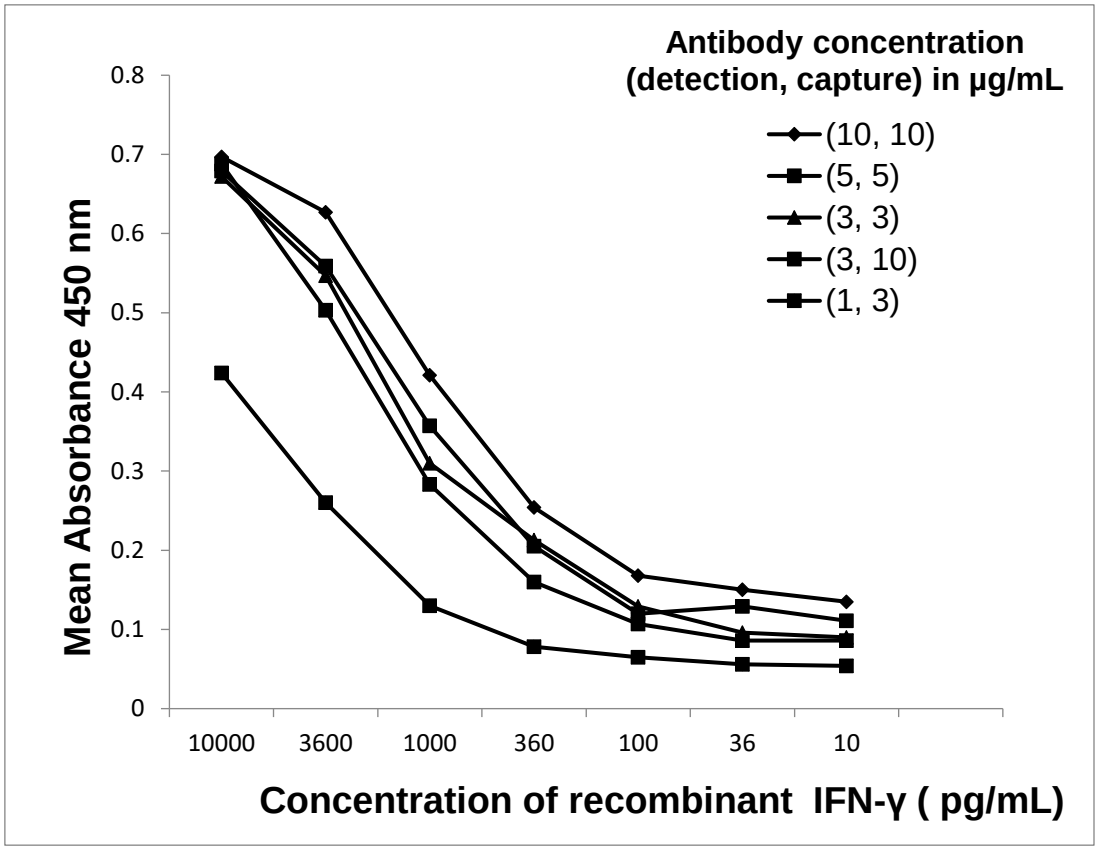

Figure 4.1 ChIFGAM ELISA development and optimization of assay conditions using serial dilutions of recombinant IFN-γ. Different combinations of detection and capture antibody concentrations were tested for an optimum assay performance, and a detection antibody concentration of 3 µg/mL and the same concentration of capture antibody was identified to be optimal to use in final assay protocols.

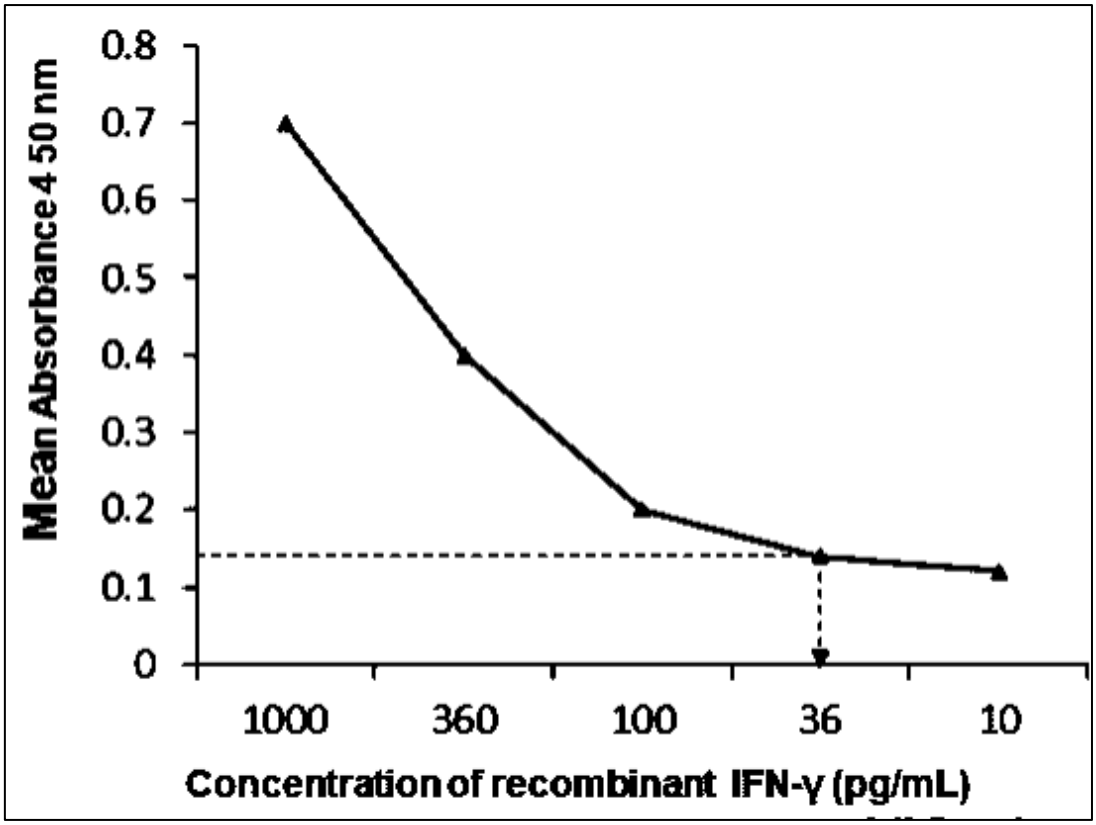

Figure 4.2 Analytical sensitivity of ChIFGAM ELISA. Twenty four replicates containing only assay diluent were tested for background signals using the ChIFGAM ELISA. The mean absorbance at 450 nm value of the replicates plus three standard deviations was 0.13 with a coffiecient of variation of 3%. The lowest concentration of IFN-γ detectable using the ELISA was defined as 36 pg/mL of recombinant IFN-γ protein using the standard curve (broken line).

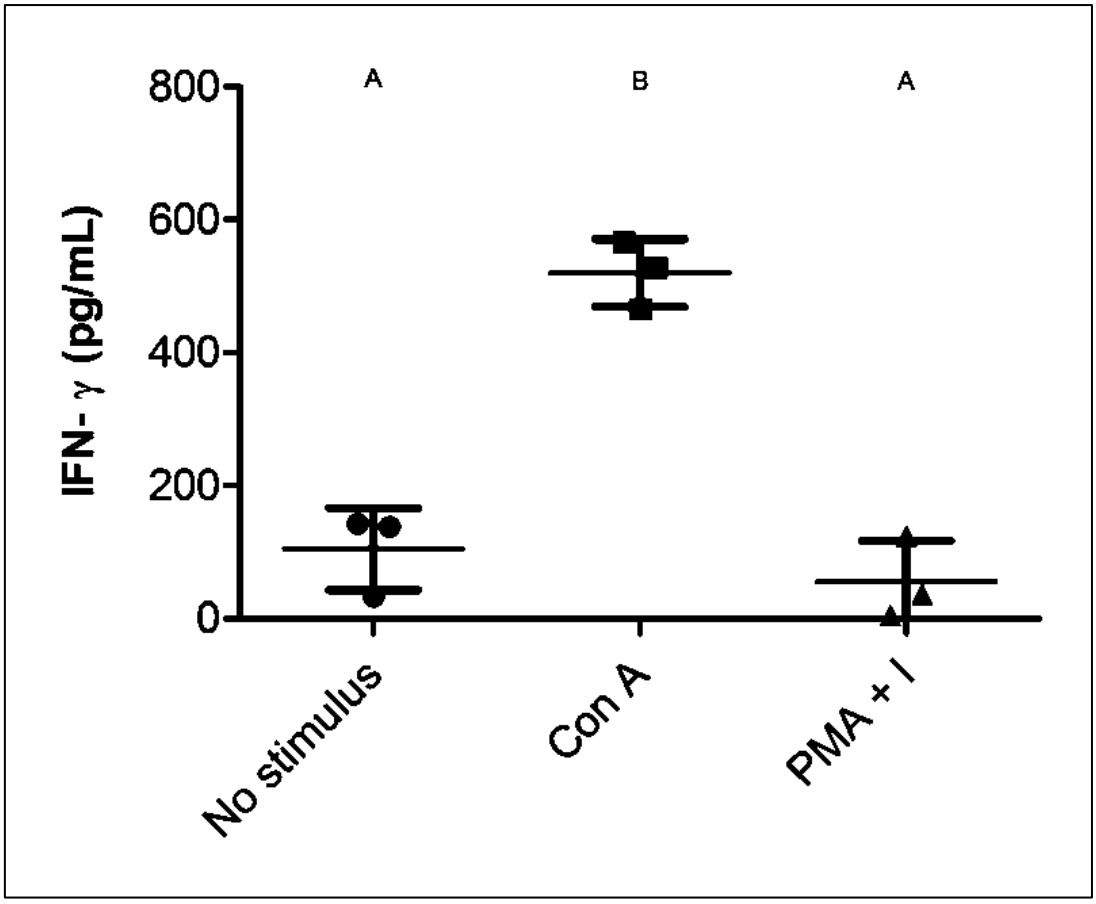

Figure 4.3 Detection of chicken IFN-γ using ChIFGAM ELISA after in vitro mitogen stimulation of splenocytes. Spleens from 3 birds were pooled and the resulting splenocyte suspension was stimulated in triplicate for 48 h with medium (no stimulus), Con A 40 µg/mL or a PMA + I at 100 ng/mL and 500 ng/mL respectively. Values with similar uppercase letter (A, B) were not significantly different (P > 0.05, Student's *t*-test). Each data point represents a well, mean values are shown and error bars indicate standard deviations.

4.3.2 Application of the splenocyte stimulation assay and the ChIFGAM ELISA to detect ILTV antigen specific CMI responses in ILTV infected birds

To examine the suitability of the assay as a potential tool for assessing CMI responses in ILTV infected birds, an *in vivo* infection study was performed to obtain splenocytes from ILTV infected birds and from matched mock-infected birds. Splenocytes were obtained from 3 groups of birds 4 weeks after infection with class 9 ILTV, or the vaccine strain SA2 ILTV or media alone as a negative (uninfected) control group. Splenocytes were purified from individual birds and used in the splenocyte stimulation assay where they were incubated with media alone, Con A (positive control for IFN-γ production), semi purified ILTV (class 9) antigen or the negative culture control antigen LMH. Splenocytes obtained from both infected and uninfected groups produced IFN-γ during co-culture with the semi purified ILTV antigen stimulation. None of the groups produced high levels of IFN-γ in response to co-culture with the negative culture control antigen LMH. The amount of IFN-γ produced following ILTV stimulation in birds previously primed with SA2 ILTV was significantly lower ($P < 0.05$) compared to those detected in mock infected birds and in birds inoculated with class 9 ILTV after *in vitro* splenocyte stimulations. Unexpectedly, Con A stimulation did not result in significant expression of IFN-γ in this experiment. This may have been due to deterioration of mitogenicity during storage (Figure 4.4).

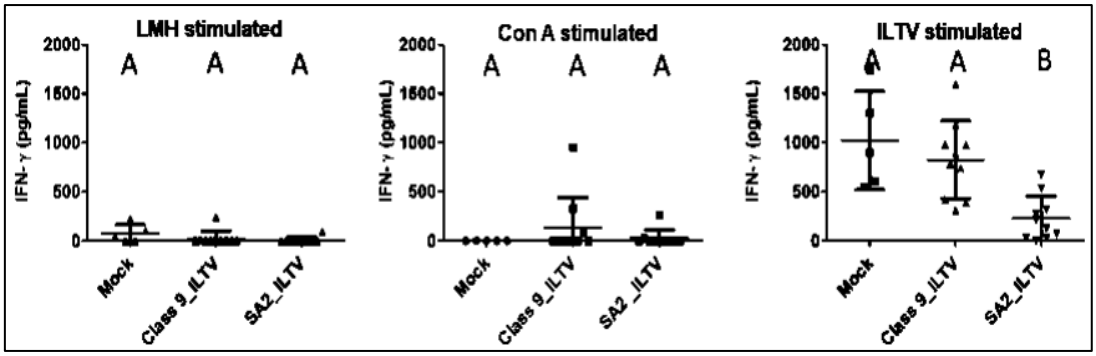

Figure 4.4 Scatter plots showing IFN-γ detection after in vitro stimulation of splenocytes by ILTV. Chicken splenocyte cultures obtained from mock-infected (n = 5), class 9 ILTV infected (n = 10) and SA2-ILTV infected (n = 10) birds were stimulated in triplicate for 48 h in culture medium either with LMH (negative control antigen, left panel) or Con A 40 µg/mL (middle panel) or semi-purified class 9 ILTV antigen (right panel) grown in LMH cells. Results are presented as mean values of individual birds with error bars indicating standard deviation. Values with the same uppercase letter (A, B) in each panel were not significantly different (P > 0.05, Student's *t*-test).

4.3.3 Inhibition of IFN-γ production by LMH and ILTV antigens during splenocyte stimulation

The results shown in Figure 4.4 did not support the hypothesis that the level of IFN-γ produced by ILTV stimulation of splenocytes from previously infected birds would be higher than that produced from splenocytes from uninfected birds. In contrast these results have shown that the presence of ILTV antigen appeared to decrease the amount of IFN-γ produced by these cells (Figure 4.4, right panel).

In order to determine whether the ILTV antigen, or the infectious nature of the ILTV antigen, is reducing IFN-γ production, an assay was performed to compare IFN-γ production by Con A stimulated splenocytes in the presence of the ILTV antigen (Figure 4.5, right panel). Compared to Con A stimulation alone, co-incubation of Con A with either the ILTV or the LMH (negative antigen) resulted in a significant reduction in IFN-γ production by the splenocytes. The finding that both the LMH and ILTV antigens reduced IFN-γ levels suggests that it is not the infectious nature of the ILTV antigen that is affecting IFN-γ production. The results from UV inactivation of the ILTV antigen also support this hypothesis (Figure 4.5, left panel). Instead, components of LMH culture themselves appear to be contributing to the inhibition of IFN-γ production seen in this system.

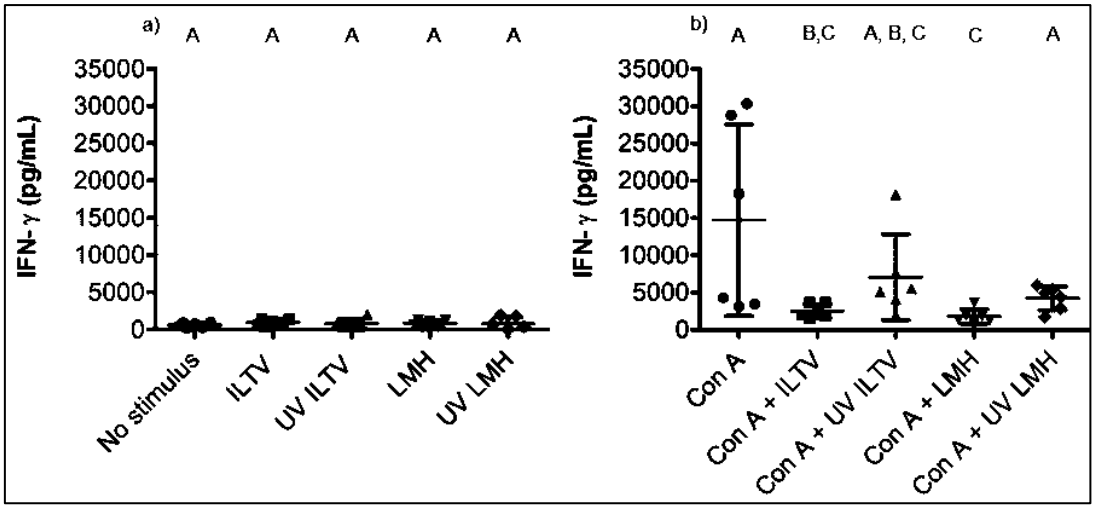

Figure 4.5 Detection of IFN-γ using CHIFGAM ELISA following splenocytes stimulations using different conditions.Splenocytes without (left panel) or with Con A (right panel) were co-incubated with untreated or UV-treated semi-purified ILTV antigen (ILTV and UV ILTV, respectively) or with untreated or UV-treated LMH antigen (LMH and UV LMH, respectively). Mean values are shown and error bars indicate standard deviations. Values with the same uppercase letter were not significnantly different (P > 0.05, Student's *t*-test).

4.4 Discussion

Monitoring flocks for immunity post ILTV vaccination is an integral part of designing disease control strategies and field vaccination protocols. Currently, the success of ILTV vaccination programs is monitored by detecting a rise in antibody titres in the serum of vaccinated animals using antibody detecting ELISA kits (Bauer et al., 1999; Chen et al., 2011; Rodríguez-Avila et al., 2008; Shil et al., 2012), despite a lack of correlation between the level of vaccine-induced protection and serum antibody titres. Little attention has been given to develop a suitable tool to measure CMI responses to ILTV vaccination (Ariaans et al., 2008; Coppo et al., 2013b) and measurement of CMI response has been described to be challenging (Coppo et al., 2013a; Lambrecht et al., 2004). This is despite the well-recognized importance of CMI responses in the protection against ILTV infection and subsequent disease (Coppo et al., 2018; Fahey et al., 1983; Fahey and York, 1990; Honda et al., 1994a).

Identifiying a suitable correlate of protection after ILTV vaccination is required to better assess the susceptibility of individuals and populations to infection and disease, and thereby better control disease. Production of antigen specific IFN-γ is one potential correlate of protection that could be suitable for this purpose. In pigs, production of antigen specific IFN-γ has shown a positive correlation with

protection against swine fever virus challenge (Suradhat et al., 2001). This is of particular interest as pig production systems typically use vaccines delivered by indirect methods such as via spray or drinking water, as occurs in poultry production systems. The level of antigen-specific IFN-γ production has also been shown to have a positive correlation with the magnitude of CMI responses in a variety of other disease models ((Andersen et al., 2017; Yin et al., 2013; Chen et al., 2011; Ariaans et al., 2009; Schiller et al., 2009; Lambrecht et al., 2004). This chapter made attempts to develop a suitable assay to detect ILTV-specific CMI responses.

To this end, first a sensitive ChIFGAM ELISA was developed using commercially available reagents. The analytical sensitivity of this ELISA was as low as 36 pg/mL of recombinant chicken IFN-γ protein. This detection limit was lower than that previously described for chicken IFN-γ ELISA, which was 50 pg/mL of chicken IFN-γ (Lambrecht et al., 2004). This previously developed ELISA used reagents (antibodies) that were no longer available. The detection limit of the assay was sufficient to detect IFN-γ production after *in vitro* splenocytes stimulation with the mitogen Con A. However, although IFN-γ production was detected after culturing splenocytes in the presence of Con A, the assay was not successful in detecting ILTV-specific IFN-γ produced after stimulation of splenocytes with semi-purified ILTV antigen. Splenocytes from ILTV infected birds would have a population of ILTV-specific T cells, which would be primed during *in vivo* infection and able to produce IFN-γ as a recall response during *in vitro* co-incubation with ILTV antigen. The amount of IFN-γ produced in such

antigen recall stimulations would be expected to reflect the magnitude of antigen specific CMI activity *in vivo* ((Abbas and Lichtman, 2005); Suradhat et al., 2001). Contrary to this expectation, ILTV specific IFN-γ production was not detected in the birds previously infected with ILTV. This could be related to the level of purity or the derivation of the recall ILTV antigen. In the future, the use of different cells such as CEK cells, or propagation of ILTV in the allantoic fluid of eggs may help to overcome the LMH substrate induced-inhibition of IFN-γ production. Alternatively, recombinant T cell immunodominant antigens could be expressed and purified for this purpose. Recall antigens generated using these methods may be less inhibitory to IFN-γ production and thus be able to induce ILTV specific IFN-γ production, and by extension help to quantify ILTV specific CMI responses. In this study whole viral antigen was prepared to make use of all possible ILTV epitopes present in the virus, since immunodominant specific ILTV epitopes have not been defined. Another reason for the use of whole virus in the current study was to take advantage of active uptake of the antigen via non-productive infection of antigen presenting cells.

The lack of specific IFN-γ detection in ILTV immune birds in the present study could also be due to insufficient concentration of ILTV specific cells to produce a detectable level of IFN-γ after short period of recall stimulation (48 h). Con A would stimulate all T cells by binding T cell receptor complexes regardless of specificity and activate them in similar ways to receptor-major histocompatibility complexes on antigen presenting cells (Abbas and Lichtman, 2005). Thus, all or many T cells would be likely to produce IFN-γ when stimulated by Con A. ILTV

antigen, however, would stimulate only a sub-population of T cells, even in a recall setting, and so fewer cells would be stimulated, thus producing smaller amounts of IFN-γ, perhaps below detectable levels. Future studies may consider the introduction of an *in vitro* amplification step to clonally expand precursors of ILTV specific T cells before actual recall stimulation assay, as has been performed for detection of CMI to other pathogens including equine influenza (Chudley et al., 2014; Paillot et al., 2007). This may increase the frequency of ILTV specific T cells, which could enable the detection of ILTV specific CMI using the ELISA assay described in this chapter.

Immunohistochemical staining has also been used to characterize chicken CMI responses to ILTV. Vaccination with gG-deficient ILTV strain (ΔgG ILTV) strain has resulted in an increased number of $CD4^+$ and $CD8^+$ T cells in the tracheal sections of birds that received the ΔgG ILTV strain compared to birds inoculated with wild type ILTV (Devlin et al., 2010). Although such assays are important for characterization of CMI responses, they are not suitable for routine immune monitoring, and also lack the capacity to measure the specificity of T cell response. In other studies, a colorimetric and fluorometric assay has been used to assess chicken CMI responses after mitogen stimulation of T cells (Gogal et al., 1997). Although such assay are simple and safe compared to radioactive isotope ([3H] thymidine) incorporation assay, which is considered a gold standard method for chicken CMI evaluation (Gogal et al., 1997), all proliferating cells are not necessarily responder T cells, thus showing a lack of correlation between lymphocyte proliferation and CMI activities (Lambrecht et al., 2004).

In order to have real utility as a screening tool to measure ILTV specific CMI in birds, use of another cell type, other than splenocytes, would be preferable. In this chapter splenocytes were chosen as they provide a concentrated source of lymphocytes. The concentrated and mixed cell populations obtained from spleen tissue may enhance the interaction between antigen presenting cells and responder T cells, and this is important for optimization of ILTV specific stimulation protocols. Future work would ideally examine the use of different cell sample types, including whole blood or PBMCs from whole blood. In cattle, the detection of IFN-γ after specific antigen stimulation of immune cells using whole blood has been successful for early screening of infection with Mycobacterium bovis, when used in parallel with skin tuberculin testing (Rothel et al., 1992; Schiller et al., 2009). Whole blood culture blastogenic responses of chickens to Con A mitogen stimulation has been documented (Lee, 1978; Talebi et al., 1995) supporting the future exploration of whole blood cultures for measuring ILTV specific CMI responses, using either IFN-γ or another potential alternative cytokine as an indicator. Moreover, alternatively assay formats such as flow cytometry and enzyme linked immunosorbent assay (Elispot) assays may also be explored in future studies.

5. General discussion

This project studied tools that may help to control disease due to ILTV, focusing on the ΔgG ILTV vaccine, and development of an IFN-γ ELISA in an attempt to measure protective cell-mediated immune responses following ILTV vaccination. These tools were selected to address key issues that are known to limit the control of ILTV in the field, including limitations associated with currently available vaccines, and the difficulties associated with measuring protective immune responses following vaccination.

Currently two types of vaccines are commercially available to help control ILT: attenuated vaccines and virally vectored recombinant vaccines (García, 2016). Attenuated vaccines have some well-documented drawbacks (Bagust, 1986; Coppo et al., 2013b; García, 2016; Garcia et al., 2013; Guy et al., 1991; Lee et al., 2012), and can have limitations when used in disease control programs (Devlin et al., 2006b; García, 2016). Recombinant virally vectored vaccines were developed to help address some of these limitations and have been released for commercial use in some geographical areas (Davison et al., 2006; García, 2016; Johnson et al., 2010; Vagnozzi et al., 2012). Several studies that have tested the suitability of these vectored vaccines have shown that they induce a relatively

poor level of protection compared to attenuated vaccines (Davison et al., 2006; Johnson et al., 2010; Sun et al., 2008; Vagnozzi et al., 2012). The reasons behind this are unclear but could include that the site of vector replication is outside the respiratory tract (Coppo et al., 2013b), or that the immunogenic potential of individual ILTV proteins is relatively weak and that a larger repertoire of ILTV specific immune responses is needed to induce high levels of protection (Stanfield and Kousoulas, 2015). Construction of recombinant virally vectored vaccines using well defined T cell determinants of ILTV may improve the capacity of future vectored vaccines to induce strong and relevant immune responses that limit replication of wild type virus and prevent subsequent clinical disease.

The development of gene deleted ILTV strains for use as attenuated vaccines has progressed alongside the development of vectored ILTV vaccines, although currently none of these deletion mutant vaccines are in commercial use. Several attempts have been made to generate attenuated mutant ILTV strains after deletion of virulence genes using recombinant DNA technologies (Devlin et al., 2006b; Devlin et al., 2007; Fuchs and Mettenleiter, 2005; García et al., 2016; Pavlova et al., 2010). One of these deletion mutant vaccines is the ΔgG ILTV examined in this thesis (Coppo et al., 2011; Devlin et al., 2008; Devlin et al., 2007). The suitability of ΔgG ILTV strain as a potential alternative live vaccine has been studied previously using different routes of inoculation and different challenge models (Coppo et al., 2011; Devlin et al., 2008; Devlin et al., 2007; Korsa et al., 2015; Legione et al., 2012; Shil et al., 2012). Together these past studies have shown that the ΔgG ILTV strain has desirable characteristics for use

as a vaccine, although a number of key questions remain to be answered. Parts of this thesis focused on addressing some of these questions, including assessing the suitability of the vaccine when delivered via drinking water under conditions resembling field conditions, and also the determination of a minimum effective dose of the vaccine when delivered via eye-drop. These studies were undertaken to enhance our understanding of the ΔgG ILTV vaccine strain and to contribute towards assessing the suitability of the candidate strain as a commercial vaccine.

Mass vaccination is an integral part of ILT control programs in large scale production systems, where inoculation of individual birds is impractical. Drinking water and eye-drop are the two commonly used routes of vaccine delivery for large scale flocks, and the route of vaccination has been reported to influence vaccine efficacy (Fulton et al., 2000). Previous studies have documented the suitability of the ΔgG ILTV strain for mass vaccination using different routes of vaccine delivery (Coppo et al., 2011; Devlin et al., 2008; Devlin et al., 2007; Legione et al., 2012). In a preliminary study by Devlin et al. (2008), birds inoculated with the ΔgG ILTV strain via eye-drop or drinking water were protected against virulent challenge. However, the study had some limitations, as it only involved a small number of birds and did not include a commercial vaccine as a comparator for vaccine performance. Additionally, the study only used SPF (layer) birds, while under field conditions drinking water is frequently used to deliver ILTV vaccines to broiler chickens. The studies described in this thesis investigated the safety and efficacy of the ΔgG ILTV strain, compared to

commercial attenuated vaccines, when administered via drinking water to broiler chickens using challenge with recombinant class 9 strain to assess efficacy. All vaccines induced protection against recombinant virulent class 9 virus, supporting previous findings (Devlin et al., 2008), but none of the vaccines induced complete protection against challenge. Vaccines that induce incomplete protection have been termed “leaky” or “imperfect” vaccines and such vaccines may facilitate the emergence of virulent viruses that spread to unvaccinated chickens and cause severe disease (Devlin et al., 2016; Read et al., 2015). As all vaccines performed similarly when delivered via drinking water, it is possible that the method of delivery limited the ability of the host to generate protective immune responses. This may be because vaccination via drinking water delivers vaccine mainly to the gastrointestinal tract, rather than the respiratory tract, where ILTV is able to replicate to high levels (Bagust et al., 2000; Coppo et al., 2012; Devlin et al., 2008; Devlin et al., 2007; Fulton et al., 2000; Robertson and Egerton, 1981). Future studies focusing on effectively delivering the vaccine via a method that allows the vaccine to reach the sites of viral replication, particularly the respiratory tract, are warranted. These more appropriate delivery methods include eye-drop vaccination, and potentially delivery via spray or aerosol.

Eye-drop delivery of the ΔgG ILTV vaccine strain was selected for further study in the studies described in this thesis. This method of delivery allows virus to reach sites of ILTV replication, including the conjunctiva and the upper respiratory tract (Coppo et al., 2011; Devlin et al., 2008; Fulton et al., 2000; Robertson and Egerton, 1981; Rodríguez-Avila et al., 2007; Sinkovic and Hunt, 1968). When

delivered via eye-drop, the ΔgG ILTV strain induced a high level of protection against challenge, at a lower dose than was used for drinking water vaccination. The lowest effective dose that protected birds against virulent challenge when delivered via eye-drop was $10^{3.8}$ PFU. This is a commercially feasible dose for vaccine production in SPF eggs and is a promising result for further development of this vaccine strain into a commercial product. It is interesting to note that the level of protection did not increase when the dose of vaccine delivered via eye-drop was increased from $10^{3.8}$ PFU to $10^{5.0}$ PFU. In contrast to the eye-drop delivery study, the drinking water delivery study examined only one dose of the ΔgG ILTV vaccine strain ($10^{5.0}$ PFU). Future studies are warranted to determine if a lower dose of vaccine delivered via drinking water can be used to induce a similar level of protection to that achieved using the high dose of $10^{5.0}$ PFU, as this would help to select the most economical dose for future commercial use. The ability of the ΔgG ILTV strain to protect birds against virulent recombinant class 9 challenge had not been investigated prior to the initiation of the studies described here. Its ability to induce a high level of protection when delivered via eye-drop, and a level of protection equivalent to that provided by the current commercial vaccines when delivered via drinking-water, is promising for its potential future use as a commercial product, including in Australia where class 9 and other virulent recombinant viruses are responsible for many outbreaks of disease in commercial poultry (Agnew-Crumpton et al., 2016; Lee et al., 2014a; Lee et al., 2012).

An additional factor that could contribute to the suitability of the ΔgG ILTV strain as a vaccine is its ability to augment CMI responses, rather than humoral immune responses, in vaccinated chickens (Coppo et al., 2018; Devlin et al., 2010). This could be advantageous, as CMI, rather than humoral immunity, is more protective against ILTV infection and disease (Chen et al., 2011; Coppo et al., 2018; Fahey et al., 1983; Honda et al., 1994b). Assessing CMI responses can be more difficult than assessing antibody-mediated immune responses, but for ILTV it would be particularly useful to examine CMI responses after vaccination as a potential correlate of protection against disease. Such an approach could be used to improve disease control programs in the field by enabling the effectiveness of vaccine programs to be assessed, and improved if necessary, before flocks are exposed to challenge with field strains of ILTV (Chen et al., 2011; Coppo et al., 2013a; Coppo et al., 2013b). This approach would be relevant for the ΔgG ILTV vaccine strain, but also for other ILTV vaccines.

At the commencement of the studies described here, antibody ELISA kits were the only available tool to screen flocks for immunity after vaccination (Bauer et al., 1999; Coppo et al., 2013b; Fulton et al., 2000; Rodríguez-Avila et al., 2008; Sander and Thayer, 1997; Shil et al., 2012; Tong et al., 2001). However, as antibodies are not protective against ILTV by themselves (Devlin et al., 2007; Fahey et al., 1983; Fahey and York, 1990), these antibody ELISAs do not accurately reflect the immune status of the flocks or individuals after ILTV vaccination. To address these limitations, this project sought to develop an ELISA to quantitate CMI responses (specifically IFN-γ production) to ILTV vaccination

using an *in vitro* antigen recall stimulation assay. The optimised ELISA was capable of accurately detecting small quantities of recombinant chicken IFN-γ protein. Although the assay was capable of detecting IFN-γ production by splenocytes after *in vitro* stimulation with a positive control mitogen (Con A), ILTV specific production of IFN-γ was not increased when splenocytes collected from ILTV-infected birds were recall activated *in vitro*, compared to those from uninfected birds.

Production of IFN-γ after specific antigen recall activation of whole blood cultures obtained from infected and uninfected animals has been used as an indicator of CMI responses in various food animal species (Parida et al., 2006; Rothel et al., 1992; Schiller et al., 2009). The Bovigam kit (ThermoFisher Scientific) has been used successfully with whole blood samples, in parallel with skin tuberculin testing for the early detection of *Mycobacterium bovis* infection in cattle (Rothel et al., 1992; Wood and Rothel, 1994). Although this current study attempted to assess IFN-γ production from splenocytes, antigen recall activation of whole blood culture may be expected to provide several advantages over splenocytes or PBMC-based assay formats in terms of measuring CMI activities. Whole blood is simple to collect, and the assay maintains the appropriate physiological environment where cellular interactions are preserved, potentially resulting in less intra-assay variation and minimal technical requirements for handling the sample. The results obtained from such assays could provide a better picture of the host's immune status (Lagrelius et al., 2006; Lee, 1978; Silva et al., 2013), raising the

possibility of establishing such an assay in order to study CMI responses during ILTV infection.

Further exploration of splenocyte cultures may also be worthwhile. Splenocyte cultures with specific antigen recall stimulation have been used successfully to measure CMI responses against some other avian pathogens (Ariaans et al., 2008; Lambrecht et al., 2004; Yin et al., 2013). It is possible that further optimisation and future improvements to the protocols developed in this current study would help to produce more consistent results. Alternatively, despite the success of IFN-γ based assays in other species or for other infectious agents, it is possible that another indicator of CMI may be more appropriate for assessing responses to ILTV vaccination and may correlate better with subsequent protection against ILTV infection and disease. Such future work, along with the continued development of the ΔgG ILTV vaccine, and other novel ILTV vaccines, should be progressed in order to provide producers with the tools required to better control ILTV.

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