Development of a novel recombinant vaccine and
diagnostic tools for control of infectious
laryngotracheitis

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ABSTRACT

Infectious laryngotracheitis virus (ILT V) causes upper respiratory tract disease in chickens worldwide. Despite the widespread use of live attenuated ILTV vaccines, outbreaks of ILTV continue to occur in poultry flocks. New recombinant ILTV vaccines have the potential to improve disease control and also allow for a DIVA (Differentiating Infected from Vaccinated Animals) approach to disease control. This work describes the development of novel DIVA diagnostic tools to use in conjunction with a candidate ILTV vaccine deficient in glycoprotein G (ΔgG ILTV). This work also examines the suitability of ΔgG ILTV for use as a vaccine vector to express infectious bronchitis virus (IBV) genes.

Two DIVA diagnostics were developed to detect and differentiate birds vaccinated with ΔgG ILTV from those infected with wild type (WT) ILTV. Initially, an enzyme linked immunosorbent assay (ELISA) was developed using recombinant ILTV gG as the coating antigen. This ELISA was successful in detecting serum antibodies to ILTV gG in vaccinated commercial birds and could differentiate between them and unvaccinated birds. The results were less satisfactory when sera from specific-pathogen-free (SPF) chickens were tested in this ELISA. It will be useful to re-evaluate the ELISA with serum samples from commercial birds vaccinated with ΔgG ILTV. Such sera will be available when the ΔgG ILTV vaccine is approved for use in field trials in future.

The second DIVA diagnostic test developed in this body of work was a TaqMan qPCR assay for detection and differentiation of ΔgG ILTV from WT ILTV. The assay was highly specific, sensitive and reproducible and had a detection limit of at least 10 viral copies. Both the individual and multiplex formats of the PCR generated satisfactory
results when tested with laboratory samples or clinical samples originating from birds vaccinated or infected with ΔgG ILTV or WT ILTV. In the multiplex format, the assay was able to detect both ΔgG ILTV and WT ILTV in samples collected from cell cultures or eggs co-infected with both viruses.

In order to evaluate the potential of ΔgG ILTV as a recombinant vaccine, partial S1 and N genes of IBV were cloned and expressed in ΔgG ILTV. The recombinant ΔgG ILTV (rILTV-IBV) was characterised in-vitro and in-ovo. The growth characteristics of the virus in these substrates were comparable to that of the parent virus, ΔgG ILTV. The capacity of the rILTV-IBV to protect chickens against IBV challenge was investigated in-vivo by vaccinating SPF chickens with rILTV-IBV via eye-drop or intra-tracheal routes, followed by challenging with an overdose of IBV Vic-S. Results from this experiment revealed that inoculation with rILTV-IBV did not produce a detectable level of serum antibodies to S1 of IBV, nor did it confer protection from tracheal lesions induced by IBV challenge.

This has been the first reported study on development of DIVA diagnostics for use in conjunction with a gene deleted marker vaccine of ILTV. This study has also shown that the gG gene deficient ILTV has the potential to be used as a vaccine vector to express foreign antigens from another avian pathogen.
DECLARATION

The work presented in this thesis was performed in the School of Veterinary Science, The University of Melbourne, Australia. All scientific work described was performed by the author except where due acknowledgement has been made in the text. This thesis complies with The University of Melbourne requirements in being less than 100 000 words in length, excluding tables, figures and bibliography.

Scientific papers and conference presentations associated with this thesis are listed below.

Shil NK, Markham PF, Noormohammadi AH, O'Rourke D, Devlin JM (2012). Development of an enzyme-linked immunosorbent assay to detect chicken serum antibody to glycoprotein G of infectious laryngotracheitis virus. Avian Diseases 56: 509-515

Shil NK, Noormohammadi AH, Devlin JM, Markham PF. Development and validation of TaqMan real-time polymerase chain reaction assays for the quantitative and differential detection of wild type infectious laryngotracheitis viruses from a glycoprotein G deficient candidate vaccine strain. (Manuscript in preparation)

Shil NK, Noormohammadi AH, Markham PF, Devlin JM. The use of glycoprotein G deficient infectious laryngotracheitis as a vaccine vector to express antigen from infectious bronchitis virus. (Manuscript in preparation)

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# TABLE OF CONTENTS

Abstract ........................................................................................................................................... i
Declaration ...................................................................................................................................... iii
Acknowledgement ........................................................................................................................... v
List of figures .................................................................................................................................. xi
List of tables ................................................................................................................................... xii
List of abbreviations ....................................................................................................................... xiv

## 1 Review of the literature ........................................................................................................ 1

### 1.1 Introduction .................................................................................................................. 1

### 1.2 Infectious laryngotracheitis virus .................................................................................. 1

#### 1.2.1 Virus properties ....................................................................................................... 1

#### 1.2.2 Virus replication and propagation ............................................................................. 2

### 1.3 Clinical disease ............................................................................................................. 3

#### 1.3.1 Infection and transmission ....................................................................................... 3

#### 1.3.2 Clinical disease and pathology .................................................................................. 3

### 1.4 Diagnosis ....................................................................................................................... 5

#### 1.4.1 Histopathological examination ................................................................................. 5

#### 1.4.2 Detection of virus ...................................................................................................... 5

#### 1.4.3 Detection of antibody ............................................................................................... 9

### 1.5 Vaccination to control ILT .......................................................................................... 10

#### 1.5.1 Modified live vaccines ............................................................................................ 10

#### 1.5.2 Inactivated vaccines ............................................................................................... 12

#### 1.5.3 Recombinant vaccines ............................................................................................. 12

### 1.6 Infectious bronchitis ..................................................................................................... 17

#### 1.6.1 Diagnosis ................................................................................................................. 19

#### 1.6.2 Vaccination ............................................................................................................... 20
1.7 Differentiation of infected from vaccinated animals (DIVA) .......................... 21

1.8 Research aims ........................................................................................................... 23

2 Materials and methods .................................................................................................. 24

2.1 Enzyme-linked immunosorbent assay ........................................................................ 24
  2.1.1 Expression of ILTV gG antigen in **E. coli** and its purification ............ 24
  2.1.2 Expression and purification of ILTV gG antigen from insect cells .......... 31
  2.1.3 ELISA using polyclonal anti-ILTV gG antibody from hyper-immunised rats 33
  2.1.4 ELISA using sera from SPF chickens ......................................................... 34
  2.1.5 ELISA using sera from commercial chickens ............................................. 36

2.2 Multiplex **TaqMan** polymerase chain reaction assay ........................................... 38
  2.2.1 TaqMan PCR development and optimisation .............................................. 38
  2.2.2 Application of the TaqMan qPCRs to laboratory samples ......................... 42
  2.2.3 Application of the TaqMan PCRs to clinical samples ................................. 44
  2.2.4 Application of TaqMan PCR to samples co-infected with WT and ΔgG ILTV 45

2.3 Generation of recombinant ILTV expressing regions of the S1 and N genes of infectious bronchitis ............................................................................................................. 48
  2.3.1 Construction of a plasmid containing IBV gene sequences ....................... 48
  2.3.2 Co-transfection experiments ........................................................................ 52

2.4 In vitro and in ovo characterisation of rILTV-IBV .................................................. 55
  2.4.1 Cell-to-cell spread ....................................................................................... 55
  2.4.2 Growth kinetics ............................................................................................ 55
  2.4.3 Gene transcription studies ............................................................................. 56
  2.4.4 Protein expression studies ............................................................................ 58

2.5 In vivo characterisation of rILTV-IBV ..................................................................... 60
  2.5.1 Safety and vaccine efficacy of rILTV-IBV administered by eye-drop ....... 60
2.5.2 Safety and vaccine efficacy of rILTV-IBV administered by the intra-tracheal route .................................................................66

3 Development of an enzyme linked immunosorbent assay .................69

3.1 Introduction .....................................................................................69

3.2 Expression and purification of recombinant gG ..................................69

3.2.1 Expression and purification of opt gG-MBP in E. coli .......................69

3.2.2 Expression and purification of gG-His in insect cells .......................70

3.3 Development of ILTV gG ELISA ....................................................71

3.3.1 ELISA optimisation using gG-MBP as antigen ...............................71

3.3.2 ELISA optimisation using gG-His as antigen .................................72

3.3.3 gG-His ELISA testing of field sera .............................................75

3.3.4 gG-His ELISA testing of experimental SPF chicken sera ...............77

3.3.5 Reproducibility of the gG-His ELISA ........................................80

3.4 Discussion .......................................................................................82

4 Development of a TaqMan real-time polymerase chain reaction assay ....85

4.1 Introduction .....................................................................................85

4.2 Assay development and validation ..................................................85

4.2.1 Assay optimisation ......................................................................85

4.2.2 Assay specificity ........................................................................86

4.2.3 Application to clinical samples ....................................................87

4.2.4 Assay reproducibility .................................................................88

4.2.5 Application to samples co-infected with WT and ΔgG ILTV ..........91

4.3 Discussion .......................................................................................94

5 The generation and initial characterisation of recombinant ILTV expressing regions of the S1 and N gene of infectious bronchitis virus .........................98
LIST OF FIGURES

Figure 2.1 Schematic of the WT and ΔgG ILTV genome showing relative positions of primers and probes. ................................................................. 39

Figure 2.2 Generation of recombinant ILTV-IBV .................................................. 50

Figure 3.1 Expression, purification and detection of recombinant opt gG-MBP and MBP purified from E. coli cultures ...................................................................................... 70

Figure 3.2 Detection of recombinant gG-His purified from infected insect cells ....... 71

Figure 3.3 Optimisation of ELISA conditions using opt gG-MBP antigen .............. 73

Figure 3.4 Optimisation of ELISA conditions using gG-His antigen .................... 74

Figure 3.5 ELISA testing of field sera from vaccinated and unvaccinated commercial birds .......................................................................................... 76

Figure 3.6 ELISA testing of sera from experimental SPF birds ............................. 78

Figure 3.7 Reproducibility of the gG-His ELISA for testing chicken sera from commercial chickens .............................................................................. 81

Figure 3.8 Reproducibility of the gG-His ELISA for testing sera from SPF chickens .. 81

Figure 4.1 Growth curves of WT and ΔgG ILTV in LMH cells .............................. 92

Figure 4.2 Growth curves of WT and ΔgG ILTV in embryonated eggs .................. 93

Figure 5.1 Photomicrograph of the LMH cell monolayer after co-transfection ....... 99

Figure 5.2 Growth kinetics of rILTV-IBV and ΔgG ILTV in LMH cells ................. 101

Figure 5.3 Growth kinetics of rILTV-IBV and ΔgG ILTV in embryonated eggs ...... 102

Figure 6.1 Detection of anti-S1 IBV antibody ....................................................... 111

Figure 6.2 Bird mortality following intra-tracheal vaccination ............................... 120
LIST OF TABLES

Table 2.1 Primers and probes used for ILTV TaqMan PCR assay .......................... 39
Table 2.2 Primers used for construction of the rILTV-IBV ................................. 49
Table 2.3 Primers used to confirm the sequence of rILTV-IBV .............................. 54
Table 2.4 Primers used for gene expression studies ............................................ 57
Table 2.5 Experimental design to assess the safety and vaccine efficacy of rILTV-IBV delivered via eye drop .............................................................. 64
Table 2.6 Experimental design to assess the safety and vaccine efficacy of rILTV-IBV delivered by intra-tracheal inoculation .............................................. 68
Table 3.1 Contingency table showing agreement between the gG-His and Trop-ILT ELISAs using sera from ILTV-vaccinated and unvaccinated commercial birds ....... 76
Table 3.2 Contingency table showing agreement between the gG-His and Trop-ILT ELISAs using sera from ILTV-vaccinated and unvaccinated SPF birds ............... 79
Table 3.3 Contingency table for calculating the sensitivity and specificity of the gG-His ELISA in SPF birds exposed to ΔgG or other strains of ILTV ............................ 79
Table 4.1 Detection and differentiation of ILTV in laboratory and clinical samples using TaqMan qPCR .................................................................................. 89
Table 4.2 Inter-assay variations in Ct values obtained using serial dilutions of pWT-ILTV in the gG+ve TaqMan PCR ................................................................. 90
Table 4.3 Inter-assay variations in Ct values obtained using serial dilutions of pΔgG-ILTV in the ΔgG TaqMan PCR ................................................................. 90
Table 5.1 The size of plaques induced by rILTV and ΔgG ILTV in LMH cell cultures .................................................................................................................. 100
Table 5.2 Relative abundance of mRNA for IBV S1-N, ILTV UL47 and GAPDH in LMH cells infected with rILTV-IBV or ΔgG ILTV

Table 6.1 Percentage weight gain 4 days after eye-drop vaccination

Table 6.2 Percentage weight gain 28 days after eye-drop vaccination

Table 6.3 Tracheal gross and microscopic pathology scores 4 days after vaccination with rILTV-IBV

Table 6.4 Percentage weight gain 3 and 5 days after challenge with IBV Vic-S

Table 6.5 Tracheal gross pathology results 3 and 5 days after challenge with IBV Vic-S

Table 6.6 Tracheal mucosal thickness 3 and 5 days after challenge with IBV Vic-S

Table 6.7 Tracheal histopathology results 3 days after challenge with IBV Vic-S

Table 6.8 Tracheal histopathology results 5 days after challenge with IBV Vic-S

Table 6.9 Percentage weight gain 16 days after intra-tracheal vaccination

Table 6.10 Detection and quantitation of rILTV-IBV after intra-tracheal vaccination

Table 6.11 Percentage weight gain at 5 days after challenge with IBV Vic-S

Table 6.12 Tracheal gross pathology and histopathology results 5 days after challenge with IBV Vic-S

Table 6.13 Tracheal mucosal thickness results 5 days after challenge with IBV Vic-S
LIST OF ABBREVIATIONS

μg microgram
μl microliter
BAP bacterial alkaline phosphate
BDT big dye terminator
BHV 1 bovine herpesvirus 1
CAM chorioallantoic membrane
CEK chicken embryo kidney
CEL chicken embryo liver
CEO chicken embryo origin
CMI cell mediated immunity
CPE cytopathic effect
Ct copy threshold
DIVA Differentiating Infected from Vaccinated Animals
DMEM Dulbecco’s Minimal Essential Medium
DNA deoxyribonucleic acid
DNase deoxyribonuclease
dNTP deoxynucleotide triphosphate
E. coli Escherichia coli
EDTA ethylenediaminetetraacetic acid
eGFP enhanced green fluorescence protein
EID_{50} median embryo infective dose
ELISA enzyme-linked immunosorbent assay
FBS foetal bovine serum
g gram
G+C guanosine plus cytosine
gC  glycoprotein C
gD  glycoprotein D
gG  glycoprotein G
GST  glutathione-S transferase
H&E  haematoxylin and eosin
HI  haemagglutination inhibition
HPAIV  highly pathogenic avian influenza virus
HSV-1  herpes simplex virus 1
HVT  herpes virus of turkey
IB  infectious bronchitis
IBV  infectious bronchitis virus
IFA  immunofluorescence assay
ILT  infectious laryngotracheitis
ILTV  infectious laryngotracheitis virus
IPTG  isopropyl β-D-thiogalactopyranoside
IR  internal repeat domain
LB  Luria Bertani broth
LMH  leghorn male hepatoma
M  molar

*M. gallisepticum*  *Mycoplasm gallisepticum*

MBP  maltose binding protein
MDV  Marek’s disease virus
mg  milligram
min  minute
ml  millilitre
mM  millimolar
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<tbody>
<tr>
<td>MOI</td>
<td>multiplicity of infection</td>
</tr>
<tr>
<td>N</td>
<td>nucleocapsid</td>
</tr>
<tr>
<td>NDV</td>
<td>Newcastle disease virus</td>
</tr>
<tr>
<td>°C</td>
<td>degree celsius</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate buffered saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>SDS-PFGE</td>
<td>sodium dodecyl sulphate-polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PFU</td>
<td>plaque forming unit</td>
</tr>
<tr>
<td>PRV</td>
<td>pseudorabies virus</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNase</td>
<td>ribonuclease</td>
</tr>
<tr>
<td>RT</td>
<td>reverse transcriptase</td>
</tr>
<tr>
<td>s</td>
<td>second</td>
</tr>
<tr>
<td>S</td>
<td>spike</td>
</tr>
<tr>
<td>SOE</td>
<td>gene splicing by overlap extension</td>
</tr>
<tr>
<td>SPF</td>
<td>specific pathogen free</td>
</tr>
<tr>
<td>STDEV</td>
<td>standard deviation</td>
</tr>
<tr>
<td>TK</td>
<td>thymidine kinase</td>
</tr>
<tr>
<td>TR</td>
<td>terminal repeat domain</td>
</tr>
<tr>
<td>Tris</td>
<td>tris(hydroxymethyl)aminomethene</td>
</tr>
<tr>
<td>UL</td>
<td>unique long domain</td>
</tr>
<tr>
<td>US</td>
<td>unique short domain</td>
</tr>
<tr>
<td>vCKBP</td>
<td>viral chemokine binding protein</td>
</tr>
<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-indolyl-β-galactopyranoside</td>
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1 Review of the literature

1.1 Introduction

Infectious laryngotracheitis (ILT) is a significant upper respiratory tract disease of chickens with a worldwide distribution. It is common in areas of intense poultry production (Guy and Garcia, 2008). The disease may result in severe production losses in commercial layer flocks. Sporadic outbreaks also occur in broiler flocks in several countries including Australia (Blacker et al., 2011). Flocks with persistent infection (both commercial and backyard flocks) can exist alongside flocks that are free from ILT. Serious disease outbreaks occur periodically whenever ILT spreads from persistently infected flocks into flocks of unvaccinated chickens (Bagust et al., 2000). The disease was first described in 1925 and was reported in Australia in 1935 (Cover, 1996).

1.2 Infectious laryngotracheitis virus

Infectious laryngotracheitis virus (ILTV) is the only member of the Iltovirus genus within the family Herpesviridae in the subfamily Alphaherpesvirinae. The virus is taxonomically identified as Gallid herpes virus 1 (Davison et al., 2005).

1.2.1 Virus properties

Infectious laryngotracheitis virus has an icosahedral capsid surrounded by a proteinacious tegument layer and outer envelope with incorporated viral glycoproteins. Virion size varies between 200-350 nm in diameter with a constant 100 nm capsid diameter (Granzow et al., 2001). The molecular weight of ILTV DNA is $100 \times 10^6$ and the genome consists of a linear DNA approximately 155 kilobase pairs (kbp) in length with a G + C content of 48.2%. The genome has unique long (UL) and unique short
(US) regions. The US region is flanked by two inverted repeats, typical of the type D herpesvirus genome arrangement (Fuchs et al., 2007; Guy and Garcia, 2008). The ILTV genome contains 76 open reading frames (Thureen and Keeler, 2006) including 12 glycoproteins. These glycoproteins play an important role in stimulating humoral and cell mediated immune responses (York and Fahey, 1990).

1.2.2 Virus replication and propagation

The replication of ILTV appears to be similar to that of other alphaherperviruses, including human herpes simplex viruses 1 and 2 (HSV-1 and HSV-2). However, analyses of ILTV gC and gB have revealed the absence of heparin binding domains and the infectivity of ILTV in cell culture remains unchanged with additional heparin or heparinase. Thus attachment of the virus to host cells occurs in a heparin-independent manner, (Kingsley and Keeler, 1999). Viral replication occurs in the nucleus. The process of protein expression proceeds in a highly regulated, sequentially ordered cascade. Three waves of transcription occur, resulting in the formation of three groups of polypeptides, designated α (immediate early), β (early) and γ (late) proteins. All three waves of transcription are catalysed by cellular ribonucleic acid polymerase II (Costanzo et al., 1977). In a recent study, ILTV genes were re-classified as immediate early, early, early-late and late based on the abundance of transcripts during the course of infection and their dependence on de novo protein synthesis or DNA replication (Mahmoudian et al., 2012). Transcription occurs in the nucleus of the infected cell whilst protein synthesis takes place in the cytoplasm (Roizman and Knipe, 2001).

In the laboratory, ILTV can be propagated in the allantoic cavity and on the chorioallantoic membrane (CAM) of embryonated chicken eggs, or in a variety of avian cell cultures including chick embryo liver (CEL), chick embryo lung, chick embryo
kidney (CEK) and chicken kidney cell cultures. The virus has also been shown to replicate in LMH cells, a continuous avian cell line derived from a chemically induced chicken liver tumor (Schnitzlein et al., 1994). In cell culture the virus forms multinucleated giant cells (syncytia) due to cytoplasmic fusion (Guy and Garcia, 2008).

1.3 Clinical disease

1.3.1 Infection and transmission

Infectious laryngotracheitis virus infection has an incubation period of 6 to 12 days (Seddon and Hart, 1935). Upper respiratory and ocular routes are the main portals of entry and less commonly through ingestion. Infected chickens excrete virus in nasal, oropharyngeal, tracheal and conjunctival exudates and transmission occurs by aerosol or in expectorant (Bagust and Guy, 1997). Acutely infected birds are the main source of infection but mechanical transmission can occur via contaminated equipment and litter (Kingsbury and Jungherr, 1958). Viral replication occurs in the respiratory tissues including the epithelium of the conjunctiva, larynx, trachea and nasal cavity. Viremia has not been detected (Hitchner et al., 1977). The virus is able to establish livelong latency in trigeminal ganglia (Rodriguez-Avila et al., 2007). Latency can be established by both field strains and attenuated vaccine strains of ILTV (Bagust, 1986). Reactivation of latent infection and subsequent shedding of virus can occur periodically, particularly during times of stress (Helferich et al., 2007; Hughes et al., 1989).

1.3.2 Clinical disease and pathology

Disease associated with ILTV infection is characterised by acute respiratory signs includes gasping, coughing, sneezing, depression, nasal discharge and conjunctivitis. Haemorrhagic and/or mucoid exudate is common in the trachea. During outbreaks with
highly virulent strains of virus clinical signs can progress to gasping, loud coughing, marked dyspnoea, head shaking and expectoration of blood stained mucous or frank blood on the face, beak and feathers. Birds can display a raised neck and extended head during inspiration. Morbidity rates of 90-100% and mortality rates of up to 70% have been observed, although mortality rates are usually in the range of 10-20% (Beach, 1926; Linares et al., 1994; Seddon and Hart, 1935).

Gross pathological lesions are found in the upper respiratory tract and conjunctiva. The severity of the lesions varies with disease severity. Diphtheritic changes in the trachea are common and may be seen as mucoid casts that extends the entire length of trachea. Severe haemorrhage can result in blood casts in the trachea, these casts may be mixed with mucus or necrotic tissues (Linares et al., 1994).

Microscopic examination of infected respiratory tissues reveals loss of goblet cells, infiltration of inflammatory cells and formation of multinucleated cells in the tracheal mucosa during the early stage of infection. This is followed by cell destruction and desquamation, producing a tracheal mucosal surface with a thin layer of basal cells, or lacking any epithelial covering. Intranuclear inclusion bodies appear within three days of infection and disappear with necrosis and desquamation of epithelial cells (Guy et al., 1992; Vanderkop, 1993).

The chicken is considered the primary host of ILTV. The disease affects all ages of birds, however the most characteristic signs are observed in adult birds. Disease has also been reported in pheasants and turkeys and a subclinical infection has been observed in ducks (Portz et al., 2008; Yamada et al., 1980).
1.4 Diagnosis

Acute ILT can be provisionally diagnosed on the basis of clinical signs and typical gross lesions. In other cases one or more confirmatory diagnostic tests should be performed (Guy and García, 2008). Laboratory diagnosis can include histological examination, detection of virus or detection of antibody.

1.4.1 Histopathological examination

Infection with ILTV can be diagnosed on the basis of pathognomonic histopathological changes in the trachea, specifically the visualisation of intranuclear inclusion bodies in the tracheal epithelium (Reynolds et al., 1968). Diagnosis of ILTV infection using histopathological techniques can be expensive and time consuming, although a rapid method for histopathologic identification of ILTV has been described. This rapid technique requires as little as 3 hours for the preparation of the tissues (Pirozok et al., 1957). Diagnostic methodologies relying on the visualisation of intranuclear inclusion bodies are only appropriate in the early stages of infection (Humberd et al., 2002).

1.4.2 Detection of virus

Virus can be isolated on the CAM of embryonated chicken eggs at 9-12 days of incubation or in susceptible cell cultures using material from trachea, larynx, or conjunctiva. Pock lesions on the CAM can be seen as early as 2 days post inoculation (Bagust, 1986). Cytopathic effects in cell culture can be observed as early as 4-6 hours post infection with high a multiplicity of infection (MOI) (Guy and Garcia, 2008). Intranuclear inclusion bodies can be detected in infected cell culture 12 hours post infection with the highest concentration observed 30-36 hours post infection (Reynolds et al., 1968). Electron microscopy can be used to visualise herpesvirus virions in pocks.
on the CAM of embryonated eggs or in tracheal scrapings and exudates (Hughes and Jones, 1988; Portz et al., 2008),

Viral antigen can be detected in clinical samples using immunoperoxidase (IP) (Guy et al., 1992), immunofluorescence (IF) (Bagust et al., 1986), dot-blot hybridisation (Nagy, 1992), in situ hybridisation (Nielsen et al., 1998), enzyme linked immunosorbent assay (ELISA) (York and Fahey, 1988), polymerase chain reaction (PCR) (Alexander et al., 1998) or real-time PCR (Creelan et al., 2006). An antigen capture ELISA using monoclonal antibodies to ILTV has been developed, this ELISA could detect the antigen in tracheal exudates and is as accurate as virus isolation (York and Fahey, 1988).

1.4.2.1 PCR-based methods to detect ILTV

In many circumstances detection of ILTV using PCR based methods has a number of advantages over other diagnostic methods. Compared with virus isolation, PCR-based methods are generally more rapid and less expensive. They are particularly useful when clinical samples are heavily contaminated with bacteria. Often PCR based techniques are able to detect a higher proportion of positive samples than virus isolation or electron microscopy (Williams et al., 1994). In one study, a sample was positive for ILTV by PCR when the only virus that could be detected by growth in tissue culture was adenovirus (Williams et al., 1994).

With the continued development and improvement of DNA technologies the number of PCR based assays to reliably and rapidly detect ILTV has increased considerably in recent years. A range of PCR-based methods have now been described, some of the PCR assays developed for ILTV are capable of detecting a single ILTV infected cell, or
10 plaque forming units (PFU) in infected cell cultures (Scholz et al., 1994). Techniques using PCR have been useful for detecting latent ILTV. This has been used to demonstrate ILTV latency in the trigeminal ganglia of chickens 61 days after inoculation (Williams et al., 1992b). A number of nested PCR assays have been described for ILTV, including an assay targeting the glycoprotein E (gE) gene of ILTV. This assay has significant value for routine diagnosis and for epidemiological studies (Chacon and Ferreira, 2008). Another nested PCR has been developed for the detection of ILTV DNA; including detection in formalin-fixed, paraffin-embedded tissues (Humberd et al., 2002). A multiplex PCR technique to detect ILTV along with six other avian respiratory pathogens has been reported (Pang et al., 2002). This multiplex PCR assay was found to be versatile, rapid, and more sensitive than immunohistochemistry for the simultaneous detection of two different avian viruses, such as infectious laryngotracheitis virus and fowlpox virus (Tadese et al., 2007). Polymerase chain reaction techniques can also be conveniently used to detect adventitious agents in avian vaccines (Vogtlin et al., 1999).

A number of real-time PCR (qPCR) assays for ILTV have been reported. Recently, a SYBR Green® qPCR has been developed to quantitate ILTV based on UL15a gene detection. The assay was highly sensitive and specific in quantifying ILTV from tissues of naturally and experimentally infected birds and embryos (Mahmoudian et al., 2011). A qPCR assay using a Taqman® labelled probe for the detection and quantitation of ILTV in chickens has also been reported. The assay targeting the glycoprotein C gene (gC) of ILTV was capable of reliably quantifying ILTV directly from clinical samples. Testing samples from both experimentally infected birds and field samples, the assay could detect approximately 3.7 times more positive samples than virus isolation
The use of a hydrolysis probe (TaqMan® probe) in qPCR is considered to be more specific than non-specific DNA labels such as SYBR Green® (Sigma) (Arikawa et al., 2008). In general, TaqMan® has been considered to be more sensitive when detecting low copy numbers (< 10 copies) because of the ability to resolve the signal of a single copy of template (Wittwer et al., 1997). In assays using TaqMan® probes a single nucleotide difference in the probe sequence will prevent the cleavage event necessary to generate a reporter signal (Bookout and Mangelsdorf, 2003). Recently, a primer-probe energy transfer based (PriProET) assay and a 5’ conjugated minor groove binder (MGB) assay have been developed based on qPCR chemistries to reliably detect and identify ILTV. Both PriProET and MGB assays were able to detect 20 copies of a DNA standard per reaction. The probes designed in the assays could differentiate vaccine/vaccine-like and virulent strains (McMenamy et al., 2011).

Restriction fragment length polymorphism (RFLP) of ILTV PCR products has been described and is now used widely to detect and differentiate vaccine and field strains of ILTV (Callison et al., 2009; Kirkpatrick et al., 2006). In one recent study, a fragment of infected cell protein 4 (ICP4) was amplified using real time PCR and subsequent RFLP on the amplified product could differentiate between older ILTV isolates that were prevalent in the 1960s (prior to the availability of vaccine strains) from more recent isolates that were similar to vaccine strains (Creelan et al., 2006). Sequence differences in the ICP4 gene of ILTV have also been used successfully in other studies to differentiate field and vaccine strains of ILTV (Chacon and Ferreira, 2009). The use of PCR-RFLP has been valuable in investigations into ILT epidemiology (Blacker et al., 2011; Chacon et al., 2010). The first complete genome sequence of the Serva vaccine
strain of ILTV has been published recently and has provided an appropriate reference for comparative genomic studies of ILTV (Lee et al., 2011).

1.4.3 Detection of antibody

Antibody detection techniques to diagnose ILTV infection include serum-virus neutralisation assays (SVN), agar gel immunodiffusion (AGID) techniques, indirect fluorescent antibody (IFA) tests and ELISA based tests (Adair et al., 1985; Bagust et al., 2000). In one study, ELISA based detection of antibody was shown to have greater sensitivity than SVN, a comparable level of sensitivity to IFA and was also found to be more suitable to test a large numbers of sera (Bauer et al., 1999). Diagnostic ELISAs to detect serum antibody to ILTV frequently utilise whole virus (Meulemans and Halen, 1982; Ohkubo et al., 1988; York et al., 1983). An ELISA using *Escherichia coli* (*E. coli*) expressed ILTV glycoproteins gp60 and gE could discriminate between unvaccinated flocks and flocks vaccinated with ILTV vaccine (Chang et al., 2002). *Escherichia coli* expressed proteins are often suitable for use as ELISA antigen to detect antibodies against a variety of viral disease (Anderson et al., 1999; Ndifuna et al., 1998; Ro et al., 1995) despite a lack of post-translational glycosylation (Kakkanas et al., 1995). Advantages of using an *E. coli* expression system to generate ELISA antigen include the relatively low productions costs and general ease in purification of suitably tagged recombinant proteins (Chang et al., 2002). Disadvantages of using *E. coli* expressed antigen include high background absorbance levels when testing negative field sera. This can be due to the animals being exposed to *E. coli* proteins that may even contaminate purified preparations of recombinant proteins derived from *E. coli* cultures (Chang et al., 2002; Ro et al., 1995; Wu et al., 1999). Another cause of high background absorbance levels in ELISAs using chicken sera is the unusual affinity of
avian serum to plastic and polystyrene surfaces (Slaght et al., 1978). The age of the chicken is also related to non-specific reactions (Bauer et al., 1999).

Cellular defense mechanisms (cytotoxic lymphocytes, lymphokines) are responsible for the protection of epithelial cells in animals vaccinated with ILTV vaccines (Fahey and York, 1990). Though antibody production may serve as an easily detected measure of exposure (Sander and Thayer, 1997), ELISA antibody titer may not necessarily be correlated with protection against ILTV (Fulton et al., 2000). Therefore, ILT ELISAs can be a useful tool to determine if a flock has been in contact with field or vaccine virus, but not to evaluate the immune status of individual birds (Andreasen et al., 1990; Bauer et al., 1999). In addition, conventional ILT ELISAs cannot differentiate antibody responses induced by vaccination versus those induced by field challenge (Sander and Thayer, 1997). This is due to the antigenic homogeneity of field and vaccine strains of ILTV (Guy and Bagust, 2003). Chang et al., (2002) suggested that a single (recombinant) antigen may be useful for such differentiation if the serum lacks antibodies against that protein, as would occur in response to vaccination with gene deleted vaccine. Serum antibodies to ILTV can generally be detected by ELISA within 2 weeks of vaccination or exposure and remain high for 4-7 weeks (Sander and Thayer, 1997).

1.5 Vaccination to control ILT

1.5.1 Modified live vaccines

A method for immunisation of chickens against ILT was first devised based on application of virulent virus to the cloaca (Brandly and Bushnell, 1934). Subsequently, field strains of ILTV have been attenuated by sequential passage of virus in cell cultures (Gelenczei and Marty, 1965; Izuchi et al., 1984) or embryonated chicken eggs (Samberg
Modified live virus vaccines generate good protection from disease following application via eye-drop, intranasal instillation or oral administration through drinking water (Bagust and Johnson, 1995). In large-scale vaccination programs application of attenuated vaccines via drinking water or by spray are desirable. Administration by drinking water, however, can be problematic as a proportion of birds may fail to develop protection. Administration by spray can result in adverse reactions, particularly if the virus is insufficiently attenuated (Robertson and Egerton, 1981).

In Australia, three live attenuated ILT vaccine strains are used to control disease; SA2 and A20 ILTV (Zoetis Australia) and Serva ILTV (MSD Animal Health.). All these vaccines are chicken embryo origin (CEO) ILT vaccines. The A20 vaccine is considered safe for use in young chickens and broiler birds. The SA2 vaccine is less attenuated and is recommended for use as a booster vaccine in layer birds following administration of the A20 vaccine. Live attenuated vaccines, including vaccine strain SA2, have a number of limitations, including the ability to establish latent infection in vaccinated birds with subsequent intermittent shedding of reactivated virus. These vaccines also have the ability to spread from vaccinated to unvaccinated birds (Bagust, 1986; Bagust and Johnson, 1995; Dufour-Zavala, 2008; Samberg et al., 1971). During bird-to-bird passage vaccine virus can revert to high levels of virulence (Guy et al., 1991). Recently it has been shown that both tissue culture origin (TCO) and CEO vaccine viruses can be transmitted from vaccinated birds to in-contact birds. Viral replication in the in-contact birds reaches similar levels to those in vaccinated birds (Rodriguez-Avila et al., 2007). A number of studies indicate that many field outbreaks of ILT are caused by strains of ILTV that are indistinguishable from CEO vaccine
strains (Dufour-Zavala, 2008). Spread of vaccine viruses between flocks or production sites may be prevented by biosecurity measures, and by using vaccination methods that ensure simultaneous infection with ILT vaccine virus of all susceptible birds on a farm (Guy and García, 2008). Recently it has been shown that independent recombination events between distinct attenuated vaccines strains resulted in virulent recombinant viruses (Lee et al., 2012).

1.5.2 Inactivated vaccines

Inactivated whole-virus ILT vaccines and subunit preparations containing affinity-purified ILTV glycoproteins have been found to be alternatives to attenuated ILTV vaccines (Barhoom et al., 1986; York and Fahey, 1991). However due to the high costs associated with producing these inactivated vaccines, as well as the high costs and inconvenience of parenteral delivery, these vaccines are not considered to be suitable for immunisation of large chicken flocks (Fuchs et al., 2007).

1.5.3 Recombinant vaccines

1.5.3.1 Vectored ILT vaccines

The development of live vector vaccines is a growing area of research in the field of animal disease control (Wang et al., 2009). The success of such vaccines in poultry may be related to the ability of the vector to replicate well in the inoculated birds (Cavanagh, 2007). A number of avian viruses namely fowl poxvirus, fowl adenovirus and vaccinia virus have traditionally been used as vectors to deliver protective antigen(s) to poultry (Britton et al., 2005; Davison et al., 2006; Draper and Heeney, 2010; Johnson et al., 2003; Karaca et al., 1998). More recently, the refinement of reverse genetic technologies has allowed the rescue of attenuated RNA viruses and their use as potential
expression or delivery vectors (Brun et al., 2008). Newcastle disease virus (NDV) has successfully been used as a vector to express H5 of highly pathogenic avian influenza (HPAI) virus. A single dose of the recombinant virus in chickens induced both NDV- and AI H5-specific antibodies and protected chickens from challenge with a lethal dose of both velogenic NDV and homologous and heterologous HPAI (Ge et al., 2007). Protection has also been observed in another NDV vectored vaccine against highly pathogenic avian influenza (DiNapoli et al., 2010; Nayak et al., 2009).

A number of recombinant vaccines have been developed and tested for their ability to control ILT. These recombinant vaccines include live vaccines constructed by insertion of ILTV genes into other virus vectors including fowlpox virus, adenovirus or herpesvirus of turkeys (HVT) (Davison et al., 2006; Saif et al., 1994; Tong et al., 2001). In commercial settings, a fowlpox vectored ILTV vaccine delivered in ovo resulted in neurological signs and chick mortality from three to ten days of age over a three week period. These problems were avoided, when the vaccine was administered in ovo at 19 days of incubation instead of 18 days of incubation (Williams et al., 2010). There have been some reports that recombinant vectored ILT vaccines delivered in ovo do not reduce replication or spread of virulent virus following challenge, even though they may be effective at reducing clinical signs following challenge (Johnson et al., 2010). The onset of protection following vaccination with vectored ILT vaccines has also been reported to be delayed when compared with live attenuated ILTV vaccines (Gimeno et al., 2011).

Herpesviruses can also be used as vaccine vectors. The first herpesviruses of farm animals engineered to express heterologous antigen was bovine herpesvirus type 1 (BHV-1) (Kit et al., 1991). Among avian herpesviruses, Marek’s disease virus (MDV)
and HVT have been used as vectors to deliver antigen from other important poultry pathogens. Infectious laryngotracheitis virus has also successfully been used as a vector to express H5, H7 and N1 genes of avian influenza virus. These vaccines have been shown to induce protection against both viruses (Luschow et al., 2001; Veits et al., 2003). However, ILTV expressing N1 antigen did not protect birds against virulent challenge with avian influenza unless administered simultaneously with an ILTV vaccine expressing H5 antigen (Pavlova et al., 2009).

Multivalent ILTV vaccines expressing antigenic determinants from other important infectious agents of poultry would also be useful for assisting with disease control. Advantages of such multivalent vaccines include reduced costs of vaccine preparation and administration, and also increased time effectiveness associated with vaccination (Perkus et al., 1985). The narrow host range of ILTV would be beneficial for preventing uncontrolled spread of genetically modified virus to other species. For these reasons ILTV represents an important vector in recombinant vaccine technology (Pavlova et al., 2009).

1.5.3.2 Deletion mutants of ILTV

Another approach for developing recombinant ILT vaccines is to generate ILTV strains that have been attenuated by deletion of their virulence genes. A key advantage of using ILTV deletion mutants as vaccines when compared to ILTV vaccines strains that have been attenuated using conventional methods is their potential to be more readily differentiated from field strains. Differentiating between vaccinated and infected animals assists in disease control and eradication programs (Kit, 1990). Vaccines deficient in specific virulence factors facilitate serological methods of differentiating vaccinated from infected birds by the presence or absence of antibodies against the
respective viral gene products (Fuchs et al., 2005). Alternatively PCR-based tests focusing on the defined genomic differences between the vaccine and field viruses (encompassing the region of the genome removed from the virus) may be used. A number of different gene deficient mutants of ILTV have been developed. Many of these mutants have shown attenuation in chickens, including mutants deficient in thymidine kinase (UL23), dUTPase (UL50), UL0, UL47, glycoprotein J (gJ) and glycoprotein G (gG) (Devlin et al., 2006; Devlin et al., 2006a; Fuchs et al., 2005; Fuchs et al., 2000; Pavlova et al., 2009; Schnitzlein et al., 1995; Veits et al., 2003). More recently a glycoprotein C (gC) gene deletion mutant of ILTV has been generated and reported to be attenuated in chickens, although high doses still produced mortality (20%) but this was lower than the mortality rate in chickens infected with the wild type (WT) or gC-rescued virus (50%). The gC deletant mutant was considered to have an insufficient level of in vivo attenuation at these higher doses (Pavlova et al., 2010).

### 1.5.3.3 Glycoprotein-G deficient ILTV as a vaccine candidate

Recently, in Australia, a glycoprotein G (gG) deficient (∆gG) mutant of ILTV has been developed to study the relevance of gG in the pathogenicity of ILTV. The glycoprotein G gene is non-essential for virus replication in herpesviruses but is a virulence factor in ILTV. The gG deletion mutant of ILTV reached a similar titre to WT virus in experimentally infected chickens, but was significantly attenuated in relation to clinical signs, effect on weight gain and bird mortality. The expressions of other genes adjacent to gG as determined by quantitative PCR were not altered as compared with WT ILTV (Devlin et al., 2006a).

A number of recent studies have focused on the use of ∆gG ILTV as an attenuated vaccine. The degree of protection induced by ∆gG ILTV mutant was comparable to that
provided by the SA-2 ILTV vaccine, which is regarded as more immunogenic ILTV vaccine used in Australia. The ΔgG ILTV vaccine resulted in better protection from clinical signs of disease and adverse effect on weight gain than SA-2 ILTV and also A20 ILTV vaccines. The ΔgG ILTV vaccine has also been shown to reduce replication of WT ILTV after challenge (Devlin et al., 2007). Although intra-tracheal inoculation of this candidate vaccine of ILTV was safe and effective as a vaccine, this method of delivery is not suitable for mass vaccination. Different routes of vaccination, more suitable for large-scale use, were subsequently tested. Aerosol inoculation resulted in low level of safety and efficacy; however inoculation via eye-drop or drinking water resulted in similar levels of safety and vaccine efficacy to intra-tracheal inoculation. From this study it was concluded that, ΔgG ILTV appears to have potential for use in large-scale poultry vaccination programmes when administered via eye-drop or in drinking water (Devlin et al., 2008). Findings from a recent safety and efficacy study of ΔgG ILTV further demonstrated the mutant ILTV strain as a suitable vaccine candidate (Coppo et al., 2011). Vaccination with ΔgG ILTV was also shown to result in a minimal level of transmission of WT virus in vaccinated birds (Devlin et al., 2011). There may be potential to administer the ΔgG ILTV vaccine candidate in ovo (Legione et al., 2012).

Recently, ILTV gG has been shown to function as a viral chemokine-binding protein (Devlin et al., 2010). Viral chemokine binding proteins (vCKBP) form part of a strategy to escape the host’s immune system (Bryant et al., 2003; Costes et al., 2006). Recent in vitro and in vivo studies suggest that ILTV gG plays a key role in immune-modulation. Infection with gG-deficient ILTV results in increased numbers of T lymphocytes in the tracheal mucosa, as well as decreased numbers of B lymphocytes in the tracheal mucosa and decreased levels of serum antibody. This suggests an immune response that is
shifted away from a predominately antibody-based response towards a more cell-mediated response. This has potential advantages for vaccination as protection from ILT is conferred by a local cell-mediated immune response, rather than an antibody immune response (Devlin et al., 2010; Fahey and York, 1990).

1.6 Infectious bronchitis

Infectious bronchitis (IB) is a highly contagious, wide-spread disease affecting both meat-type and egg-laying chickens. Disease affects the respiratory tract primarily but can also affect the gut, kidney and oviduct (Cavanagh, 2001; Cook et al., 2001). Infectious bronchitis is an economically important disease causing reduced performance, diminished egg quality and quantity, susceptibility to infection with other pathogens and condemnation at processing (Ignjatovic and Sapats, 2005). Vaccination programs are used to control disease (Cavanagh and Gelb, 2008). Strains of infectious bronchitis virus (IBV) constantly diverge to create distinct and diverse sub-lineages of IBV exclusive to different regions and countries and this can create difficulties for disease control using vaccination (Ignjatovic et al., 1997).

Etiology

Infectious bronchitis virus is a group 3 Coronavirus of the Coronaviridae family and is in the order Nidovirales. In Australia, IBV strains have been classified as either subgroup 1, 2 or 3 or unrelated field strains (Hewson et al., 2009).

Virus properties

Coronaviruses are enveloped, pleomorphic viruses with a mean diameter of approximately 120 nm. The virions have large club shaped (20 nm) surface projections
which are the heavily glycosylated spike (S) glycoprotein (Cavanagh, 2007). The IBV genome consists of a positive sense, single stranded RNA. The 27.6 kb genome contain nine functional genes. Four of these genes encode structural proteins, namely the spike glycoprotein (S, 180 kDa), membrane glycoprotein (M, 26-34 kDa), the nucleocapsid protein (N, 50 kDa) and a small membrane glycoprotein E (E, 8.4-12 kDa) (Lai and Cavanagh, 1997).

The S glycoprotein is cleaved post-translationally into an amino-terminal S1 subunit (535 amino acids, 90 kDa) and a carboxy-terminal S2 subunit (627 amino acids, 84 kDa). Cleavage is performed by cellular proteases (Cavanagh et al., 1986b). The globular S1 forms a receptor binding site. The S2 subunit anchors the S1 subunit to the viral membrane (Cavanagh, 1983). The S1 subunit is responsible for serotype variation. Between different strains of IBV, sequence variation in the S1 region ranges from 2 to 25% at the amino acid level (Casais et al., 2003). The S1 protein also plays a role in infectivity and tissue tropism and displays haemagglutination activity. The S1 protein has been identified as the major inducer of protective immune responses, including cell mediated immune (CMI) responses (Cavanagh et al., 1986a). Among the structural proteins, the N protein is highly conserved in IBVs. The sequence variation between isolates, at the amino acid level, is only 2 to 6% (Williams et al., 1992a). The N protein induces the production of high titers of cross-reactive ELISA antibodies and CMI responses (Ignjatovic and Galli, 1994; Seo et al., 1997). Recombination is undoubtedly a feature of the replication and evolution of IBV (Ladman et al., 2006).

Infection and transmission
Infectious bronchitis virus is highly contagious and rapidly spreads among susceptible chickens. Infection occurs via the respiratory tract (Boltz et al., 2004). The virus displays an affinity for ciliated and mucous secreting cells, mainly of the upper respiratory tract (Dhinaker and Jones, 1997), however virus replication also occurs in many other epithelial surfaces including kidney, oviduct, testes and many parts of the alimentary tract (Cavanagh, 2003). Interestingly IBV can establish persistent infections in the kidney and other non-respiratory organs (Jones and Ambali, 1987). Virus can be shed periodically from persistently infected birds in nasal secretion and faeces (Gay, 2000).

Infectious bronchitis virus has a short incubation period of 18 to 36 hours (Cavanagh and Gelb, 2008). The clinical signs of IB include gasping, coughing, sneezing, tracheal rales, nasal discharge and conjunctivitis. Reduced weight gain and feed consumption are also observed (Cavanagh and Gelb, 2008). In laying flocks egg production can decline and soft shelled, misshapen or rough-shelled eggs may be produced (Crinion, 1972). The mortality rate generally ranges from 20 - 30% but may reach as high as 90% depending on the IBV serotype. Host factors such as bird sex, breed, age and nutritional status can also affect the severity of disease and the mortality rate (Cumming, 1969; Fabricant, 1998). Secondary opportunistic infections such as systemic colibacillosis are a common feature of virulent IBV infection in broiler chickens (Matthijs et al., 2005). Viruses genetically similar to IBV are increasingly being detected in other avian species including turkeys and ducks (Liu et al., 2005).

1.6.1 Diagnosis

Virus can be isolated in embryonated eggs but may need several blind passages before detection. Immunofluorescent assay, immunoperoxidase assay (IPA), RT-PCR or
antigen ELISA are more practical techniques to detect virus in clinical samples (De Wit, 2000). Adapted IBV strains can be cultured in chicken embryo kidney cells or chicken kidney cells however this technique is not suitable for isolation of all field viruses (Cook et al., 1976). Following virus isolation, the presence of virus antigen needs to be confirmed by PCR (Hewson et al., 2009), agar gel precipitation test (AGPT) (Lohr, 1981), IFA (De Wit et al., 1995), IPA (Naqi, 1990) or antigen ELISA (Ignjatovic and Ashton, 1996).

Molecular methods of detection and differentiation of IBV strains include RT-PCR followed by nucleotide sequencing, RFLP, high resolution melt curve analysis and DNA hybridisation (Hewson et al., 2009; Jackwood et al., 1992; Mardani et al., 2005; Song et al., 1998a). Nucleotide sequencing of the S1 gene is a commonly used technique to differentiate IBV strains. Type specific and universal primers have been developed to detect variation in the S gene of IBV (Keeler et al., 1998; Kingham et al., 2000).

Serological tests for IBV infection include virus neutralisation (VN), haemagglutination inhibition (HI) and ELISA (reviewed in De Wit, 2000).

1.6.2 Vaccination

Vaccination has been routinely practiced to control IB for over half a century (Bijlenga et al., 2004). Attenuated live vaccines are used in meat type chickens at one-day of age and are also used for priming in breeder and layer pullets (Huang and Wang, 2006). Booster vaccination of layer birds, and also of broiler birds, with a vaccine of a different serotype may give a better protection against a broader range of IBV serotypes (Cook et al., 1999). Vaccine virus may persist in various internal organs for up to 163 days or
longer, and may be periodically shed in nasal excretion and faeces. Increased virulence of live vaccine strains of IBV has been observed to occur after bird-to-bird passage (Gay, 2000; Hopkins and Yoder, 1986).

Inactivated IBV vaccines are also used to control disease. Their efficacy depends heavily on proper priming with a live vaccine. Inactivated vaccines require individual administration (Cavanagh and Gelb, 2008). A sub-unit vaccine consisting of the S1 protein has been generated and was shown to induce protection in over 50% of birds under field condition (Ignjatovic and Galli, 1994; Ladman et al., 2002). Inactivated vaccines reduce the load of virus in the respiratory tract after challenge and therefore limit virus transmission (Ladman et al., 2002).

Vectored vaccines represent an alternative approach for control of IB. Fowl adenovirus expressing the S1 protein from IBV Vic S strain (Zoetis, Australia) has been shown to provide protection following challenge with homologous (Vic) and heterologous (N1/62) virus (Johnson et al., 2003). A fowlpox virus expressing IBV S1 along with chicken interferon gamma (IFNγ) provided protection against virulent challenge with virulent IBV LX4 strain and significantly decreased the viral antigen present in renal tissue (Wang et al., 2009). Recombinant fowlpox vectored vaccines have some constraints in regards to their suitability for mass application in poultry and also their use in birds with high levels of maternal antibody. Other vector viruses such as adenovirus and herpesviruses may be more suitable in these situations (Boyle and Heine, 1993).

1.7 Differentiation of infected from vaccinated animals (DIVA)

Conventionally attenuated or inactivated vaccines are available and are effective at
controlling infectious diseases in many animal populations, however they frequently interfere with disease monitoring and surveillance based on a serological test (Draper and Heeney, 2010; Pasick, 2004). Marker vaccines such as subunit vaccines or attenuated vaccines with selective gene deletions can be used to facilitate differentiating infected from vaccinated animals (Meeusen et al., 2007). The term DIVA (Differentiating Infected from Vaccinated Animals) originated in 1999 at the Central Veterinary Institute, in the Netherlands (van Oirschot, 1999). The DIVA approach commonly utilises the detection of differentiating antibody responses induced by marker vaccine from those induced by WT pathogens (Meeusen et al., 2007). For live vaccines that are deficient in a specific virulence factor, PCR-based techniques targeting the region of the genome that differs between the vaccine and WT pathogens are also useful to differentiate between infected and vaccinated animals.

In other livestock species, marker herpesvirus vaccines have been successfully coupled with differential diagnostic tests to achieve DIVA capabilities. A gE deficient vaccine to control BHV-1 infection has successfully been used in conjunction with a gE-blocking ELISA that was developed as a companion diagnostic tool (Perrin et al., 1996). A specific PCR has also been developed to differentiate gE deficient BHV-1 and WT BHV-1 (Schyns et al., 1999). Deletion of the gE gene has also been used to enable a DIVA approach for porcine herpesvirus 1 (PRV-1) infection in swine (Pensaert et al., 2004). In combination with a differential ELISA, a gG-deficient vaccine strain of PRV-1 has been used in a disease eradication campaign in North America (Kit, 1990). A gE latex agglutination test showed a similar sensitivity to recombinant gE-ELISA for differentiating sera from swine receiving the gE deficient PRV vaccine from those infected with wildtype PRV (Yong et al., 2005).
In chickens, an ELISA using *E. coli* -expressed ILTV glycoproteins gp60 and gE has been developed and may play an important role in the control and/or eradication of ILT, if gp60 and gE ideficient ILTV marker vaccines become available (Chang et al., 2002). It has been noted that gC deficient ILTV may serve as a basis for a marker ILTV vaccine as gC-specific antibodies could be readily detected in sera of birds infected with WT ILTV (Pavlova et al., 2010).

The selection of an appropriate companion diagnostic tool (either a serologic tool or a PCR-based tool) to accompany a marker vaccine depends on factors such as the presence of antibody response to the vaccine and the phase of the disease when the diagnostic test is to be applied. Following infection with ILTV, antigen is present in tracheal tissues and secretions for 6 – 8 days (Guy and Garcia, 2008) or longer (Williams et al., 1992c). During this acute phase of infection PCR-based methods of detecting and differentiating ILTV vaccine or field strains would be suitable. At later time points, ILTV antigen in the trachea may not be detectable and so serologic based tests, such as an ELISA, may be more suitable.

### 1.8 Research aims

The first aim of the project was to develop novel companion diagnostic tools to differentiate birds vaccinated with recombinant ΔgG ILTV from birds infected with WT ILTV. Enzyme linked immunosorbent assay and PCR based techniques were examined for this purpose. The project also aimed to use ΔgG ILTV as a vaccine vector to express regions of the S1 and N proteins of IBV. The resultant virus was tested for its suitability as a vaccine candidate.
2 Materials and methods

2.1 Enzyme-linked immunosorbent assay

In order to develop a companion differential ELISA for use with the candidate ∆gG ILTV vaccine, two different forms of recombinant ILTV gG were purified and tested for use as ELISA antigens; *E. coli* codon optimised full length gG protein expressed in *E. coli* and full length ILTV gG protein expressed in a baculovirus expression system.

2.1.1 Expression of ILTV gG antigen in *E. coli* and its purification

2.1.1.1 Preparation of expression plasmid

The ILTV gG gene was genetically optimised for expression in *E. coli* (opt_gG) and synthesised (DNA 2.0) (Appendix 1). The plasmid carrying opt_gG was used to transform *E. coli* DH5α cells (Invitrogen) by electroporation using a BioRad electroporator set at 2.5 kV, 200 Ω and 250 μF. The transformation mixture was spread onto Luria Burtani (LB) agar plates containing kanamycin 50 μg/ml and incubated overnight at 37°C. On the following day a kanamycin resistant colony of *E. coli* was selected and used to inoculate LB broth containing kanamycin 50 μg/ml and then incubated overnight at 37°C with constant shaking (200 rpm). On the following day plasmid DNA was extracted using Midiprep plasmid extraction kit (Qiagen) according to the manufacturer’s instructions. Plasmid DNA carrying opt_gG was digested with restriction enzymes *BamHI* and *SalI* (New England Biolabs) according to the manufacturer’s instructions. The pMALp4x expression vector was also digested with the same restriction enzymes and then dephosphorylated using bacterial alkaline phosphatase (BAP) (Invitrogen) according to the manufacturer’s instructions. The digested DNA was separated in a 1% agarose gel and the DNA bands of expected size
(850 bp for opt gG and 6.7 kb for pMALp4x) were excised and extracted using QIAEX II kit (Qiagen) according to the manufacturer’s instructions. The DNA was quantified by measuring the absorbance at 260 nm using a NanoDrop® ND-100UV-Vis Spectrophotometer (Thermo scientific). The pMALp4x expression vector is designed to produce recombinant protein with a maltose binding protein (MBP) tag to facilitate protein purification. The opt_gG DNA fragment was ligated to digested pMALp4x with T4 DNA ligase (Promega) in ligation buffer (Promega) at 4°C overnight according to the manufacturer’s instructions.

Bacterial cells and culture

Three different strains of *E. coli* were transformed with the pMALp4x/opt_gG ligation mixture in order to compare expression efficiency between each cell type. The ligation mixture was used to transform *E. coli* strains DH5α, JM109 (Promega) or Rosetta Gami (Novagen) by electroporation (strains DH5α and JM109) or by heat-shock (Rosetta-gami). Transformants were screened by blue-white screening on LB agar plates containing ampicillin, isopropylthio-β-D-galactose (IPTG) (Promega) and 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (Xgal) (Amresco) following incubation overnight at 37°C. White colonies were selected and used to inoculate LB broth containing 100 µg/ml ampicillin. Following overnight incubation at 37°C with constant shaking, plasmid DNA was extracted using Wizard plus SV miniprep kit (Promega) according to the manufacturer’s instructions. The ILTV gG-encoding sequence of the plasmid was confirmed by DNA sequencing using both or either of the malE (GGTCGTCAGACTGTCGATGAAGCC) and pUC/M13 (CGCCAGGGTTTCCAGTCAGAC) forward oligonucleotide primers and Big Dye Terminator (BDT) version 3.1 chemistry (ABI PRISM) according to the
manufacturer’s instructions. Sequencing reactions were incubated in an iCycler Thermal Cycler (Biorad). Sequence chromatograms were examined using GENEious (Version 5.0, Biomatters, NewZealand.). Sequence alignments were performed using ClustalW (Thompson et al., 1994).

2.1.1.2 Protein purification and characterisation

Small-scale expression studies were performed to assess the ability of different E. coli strains to produce recombinant protein. A single colony of each of the DH5α, JM109 or Rosetta Gami E. coli cells containing the pMALp4x/opt_gG expression plasmid were cultured in 5 ml of LB broth and incubated with constant shaking at 37°C overnight. The overnight culture was used to inoculate 10 ml of LB broth at a dilution of 1/50 which was subsequently incubated with constant shaking at 37°C until absorbance at 600 nm (OD$_{600}$) reached approximately 0.5 or 0.9. A 500 µl sample of the culture was centrifuged at 10,000 × g for 5 min and the cells resuspended in 50 µl of phosphate buffered saline (PBS) (137 mM sodium chloride, 2.7 mM potassium chloride, 10 mM phosphate, pH 7.4) and stored at -20°C for use as uninduced control samples. Isopropylthio-β-D-galactose was then added to a final concentration of 0.3 mM and 500 µl samples of the induced cultures were collected (every hour for up to four hours for cultures induced at an OD$_{600}$ of 0.5 and only one hour after induction for cultures induced at an OD$_{600}$ of 0.9) for analyses. Cells were pelleted by centrifugation for 5 min at 10,000 × g and the pellet was resuspended in 50 µl of PBS and stored at -20°C to be used as induced samples.

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) and Duffy et al (1998). The uninduced and
induced samples were each mixed with 2 × SDS-PAGE sample buffer (10% SDS, 20% glycerol, 0.5 M Tris-HCL, pH 6.8, 1 M β-mercaptoethanol, 10% bromophenol blue) and heated at 100°C for 5 min. The proteins in the sample were separated on a 10% or 12.5% SDS-PAGE gel by electrophoresis at 180 V for 1 h. Broad range prestained protein marker (Fermentus) were used as molecular mass standards. The gel was stained with Coomassie brilliant blue R-250 (Biorad).

After SDS-PAGE the proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) using the mini-trans-blot system (BioRad) in Western transfer buffer (20% methanol, 50 mM tris-HCl, 380 mM glycine) at 100 V for 1 h. Following transfer, membranes were blocked overnight with 5% skim milk in distilled water at 4°C in preparation for immunoblotting.

To detect recombinant ILTV opt_gG-MBP the membrane was probed with a mouse MBP monoclonal (MAb) (diluted 1/2500 in PBS containing 0.05% (v/v) Tween-20 (PBS-T)) conjugated to horseradish peroxidase (anti-MBP-HRP conjugated antibody, New England Biolabs). Bound rat sera to recombinant ILTV gG (Devlin et al., 2010) was diluted 1/1000 in PBS-T and used to detect gG-MBP by immuno-blotting. Each antibody was incubated for 1 h at room temperature and washed 3 times for 10 min each in PBS-T. Rat hyper-immunised sera were detected using horseradish peroxidise (HRP) conjugated anti-rat immunoglobulin G (GE Healthcare). Proteins were visualised using enhanced chemiluminescence (ECL) substrate (Amersham) and the resultant reaction captured using a ChemiDoc gel documentation system (BioRad).

Of the three strains *E. coli* strains, Rosetta Gami cells were selected for recombinant protein expression after which a small-scale expression and purification experiment was
performed. The recombinant ILTV gG-MBP was expressed (described above) and the recombinant protein purified using amylose resin (New England Biolabs) according to manufacturer’s instruction with slight modifications. Briefly, a single colony was used to inoculate 5 ml of NZCYM broth (1% w/v NZ amine®, 0.5% w/v NaCl, 0.5% w/v yeast extract, 0.1% w/v casamino acids, 0.2% w/v MgSO₄·7H₂O) with 0.4% glucose, 100 µg/ml of ampicillin and incubated overnight at 37°C with constant shaking. The following day, a 1 ml aliquot of the overnight culture was used to inoculate 100 ml of NZCYM broth containing glucose and 100 µg/ml ampicillin. When the culture OD₆₀₀ had reached 0.9, IPTG was added to a final concentration 0.3 mM and after 1 h the cells were harvested by centrifugation at 4000 × g for 20 min at 4°C. Uninduced and induced samples were collected and stored as described above. The cells were lysed using B-PER® (Pierce Biotechnology) bacterial protein extraction reagent according to manufacturer’s instructions. Following protein extraction, a full micropipette tip of cell pellet resuspended in 500 µl of PBS and 500 µl of supernatant were collected as insoluble and soluble protein samples, respectively, and stored at -20°C. To prepare the amylose resin affinity column, 1 ml of amylose resin was carefully placed in a poly-prep chromatography column (BioRad) and washed with 10 ml of column buffer (1 M Tris Cl pH 7.4, 0.2M NaCl, 1 mM EDTA, 1 mM DTT). The supernatant from the cell lysis was then placed on the column matrix and allowed to flow through slowly. The column was then washed with 20 ml of column buffer and the protein was eluted in 1 ml of column buffer containing 10 mM maltose in five fractions of 200 µl each. Different samples from the flowthrough, wash and eluted proteins were tested by electrophoresis on a 10% polyacrylamide gel by SDS-PAGE and visualised by Coomassie blue staining. Western blotting was performed after transferring the protein to PVDF membrane and probing with anti-MBP monoclonal antibody or polyclonal
anti-gG sera which was detected using HRP conjugated anti-sera. The protein was also probed for 1 h with pooled sera from A20 ILTV vaccinated SPF chickens (Coppo et al., 2011) diluted 1/100 in PBS-T. The blot was washed 3 times for 10 min each in PBS-T and detected with HRP conjugated rabbit anti-chicken immunoglobulin G (Millipore) diluted 1/1000 in PBS-T. The concentration of the purified protein was assessed by Coomassie blue stained SDS-PAGE gel and comparison with known amounts of bovine serum albumin (BSA) (New England Biolabs) using ImageJ software (http://rsb.info.nih.gov/nih-imageJ, National Institutes of Health.).

2.1.1.3 Purification of MBP

The MBP tag protein was similarly expressed, purified and used as a control to measure any cross reaction with test sera. For this purpose, E. coli Rosetta Gami cells carrying the pMALp4x vector were grown using the same procedures as for gG-MBP described above. The purity of the MBP protein was examined in 10% SDS-PAGE gel stained with Coomassie blue and the concentration of the purified protein was determined as described above. Anti-MBP monoclonal antibodies were used to detect the size of the purified MBP by western blotting. Pooled serum from A20 ILTV vaccinated chicken was also used as described above. Reactivity was detected using chemiluminescence as described above.

2.1.1.4 ILTV ELISA using gG-MBP fusion protein as antigen

The recombinant ILTV gG-MBP was tested to assess its suitability for use as antigen in a differential ILTV ELISA as described below.

2.1.1.5 Origin of sera used in ELISA development

Serum samples from SPF chickens
Serum was collected from groups of three weeks old SPF hybrid white leghorn birds that had been eye-drop inoculated with SA2, A20 or Serva ILTV or sterile media (Coppo et al., 2011), or ΔgG ILTV (Devlin et al., 2008). Sera were collected from each group of birds 21 days after vaccination and stored at -20ºC until use. The commercial Trop-ILT ELISA (Tropbio Australia Pty. Ltd.) was used to confirm the presence of anti-ILTV antibodies in the vaccinated chickens and the absence of anti-ILTV antibodies in the mock-vaccinated chickens.

**Serum samples from commercial chickens**

Serum samples were obtained from 40 commercial broiler breeders of approximately 20 weeks of age that had been vaccinated with SA-2 ILTV. Additional information about these birds was unavailable. Negative serum samples were collected from 30 unvaccinated broiler birds of 34 days of age. The Trop-ILT ELISA was used to confirm the presence or absence of anti-ILTV antibodies of each bird from each group.

### 2.1.1.6 Optimisation of opt_gG ELISA

A series of experiments were conducted to ascertain the optimal antigen and serum concentration for use in the opt_gG antigen ELISA. Firstly, a range of recombinant protein concentrations were tested in duplicate. Following this a series of two-fold dilutions of pooled serum from five A20 ILTV vaccinated SPF chicken and pooled serum from 5 commercial chickens vaccinated with SA2 ILTV, were tested.

Each well of a Maxisorp ELISA plate (Nunc) was coated overnight at 4ºC with 100 µl 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing purified gG-MBP or MBP only. A range of recombinant protein concentrations were tested in duplicate using 8 two-fold dilutions starting at 8 µg/ml. The ELISA plate was washed four times with PBS-T
before blocking with 1% gelatin (Difco) in PBS, pH 7.2 at 37°C for 1.5 h. The plate was then washed as described above and 100 µl of test sera added. Experimentally vaccinated and unvaccinated SPF chicken sera and sera from commercial chickens were applied in two-fold dilution series in duplicate (commencing at 1/20 dilution for the SPF and 1/100 dilution for the commercial chickens), each dilution of sera was tested with each antigen concentration in a checkerboard pattern (Zheng et al., 2000). All test and control sera were diluted in diluent buffer (wash buffer containing 1% BSA, fraction V (Roche)). Additional control wells containing antigen, 100 µl of wash buffer only were included. The plate was incubated at 37°C for 1 h. After a further wash cycle, 100 µl of rabbit anti-chicken immunoglobulin G conjugated to HRP (GE healthcare) at a dilution of 1/1000 was added and the plate was incubated at 37°C for 1 h. After another wash cycle, 100 µl of ABTS substrate [Kirkegaard and Perry Laboratories (KPL)] solution was added to each well and the plate was incubated at room temperature for 30 min. The reaction was stopped by addition of 100 µl of dH₂O to each well. The absorbance of each well was read at 405 nm using a microplate reader (Labsystem Multiskan MS). The optimal concentration of antigen was determined to be 2 µg/ml using a serum dilution of 1/20 for SPF chicken sera and 1/400 dilution for field sera.

2.1.2 Expression and purification of ILTV gG antigen from insect cells

2.1.2.1 Preparation of recombinant baculovirus

Stocks of recombinant baculovirus expressing full-length ILTV gG with a histidine tag (ILTV gG-His) were available within the laboratory (Devlin et al., 2010).
2.1.2.2 Insect cells and culture

A clonal isolate of the *Spodoptera frugiperda* cell line (Sf9 cells) (Invitrogen) was cultured in Sf-900 II serum free medium (Invitrogen) at 27°C ± 0.5°C. The cells were typically cultured as adherent cells at a density of 2 - 5 × 10⁴ cells/cm² in 25 cm² or 75 cm² flasks in a closed system.

2.1.2.3 Infection with recombinant baculovirus

Recombinant baculovirus expressing ILTV gG-His was used to infect Sf9 cells. Cells were seeded at a density of approximately 4.0 - 5.0 × 10⁵ cells/ml into 75 cm² flask and incubated at 27º C ± 0.5ºC for 1 h. The cell monolayer was then infected with recombinant baculovirus and observed for 72 h or until virus-induced cytopathic effects were observed. Infected cells and supernatant were collected after three cycles of freezing and thawing and clarified at 1000 × g for 5 min at 4ºC and the supernatant was used to purify the recombinant protein. The supernatant was dialysed against lysis buffer (50 mM NaH₂PO₄, 300 mM NaCl, 20 mM imidazole, pH 8.0) overnight at 4ºC.

2.1.2.4 Protein purification and analysis

The histidine tagged gG was purified from the dialysed supernatant using nickel-nitriloacetic acid (Ni-NTA) agarose (Qiagen) and eluted from the column using elution buffer (50 mM NaH₂PO₄, 300 mM NaCl, 250 mM imidazole, pH 8.0). The purified protein was subjected to SDS-PAGE and stained with Coomassie blue to determine purity. Western blot was conducted using polyclonal rat sera to confirm the presence of the purified protein. The concentration of the purified protein was determined by comparison with known amounts of BSA following SDS-PAGE and staining with Coomassie blue using ImageJ.
2.1.3 ELISA using polyclonal anti-ILTV gG antibody from hyper-immunised rats

To determine if recombinant gG-His was suitable for use in ELISA, sera from rats hyper-immunised with gG-GST was tested and used to optimise ELISA antigen concentration.

2.1.3.1 Sera

Polyclonal anti-ILTV gG-GST antibody was collected from gG-GST hyper-immunised rats (Devlin et al., 2010). To produce the immunogen, inclusion bodies containing recombinant protein were separated by SDS-PAGE under reducing condition and the region of the gel containing recombinant protein was excised, homogenised in Freund’s complete and also in incomplete adjuvant and used to immunise rats. Serum was collected from the rats and stored in 0.5 ml aliquots at -20°C until use (Devlin et al., 2010).

2.1.3.2 Optimisation of ELISA conditions

Each well of a Maxisorp ELISA plate was coated overnight at 4°C with 100 µl 0.1 M carbonate-bicarbonate buffer (pH 9.6) containing purified gG-His. A range of recombinant protein concentrations were tested in duplicate (6 µg/ml to 0.25 µg/ml). The ELISA plate was washed four times with PBS-T before blocking with 1% gelatine in PBS, pH 7.2 at 37°C for 1.5 h. The plate was then washed as described above and 100 µl of test sera diluted in diluent buffer was added. The test sera were applied in four-fold dilution series in duplicate (commencing at 1/100 dilution), each dilution of sera was tested with each antigen concentration in a chequer board pattern. A negative control rat antisera against 3CT of Equine rhinitis B virus ERBV was applied in duplicate in the same dilution series. Additional control wells containing antigen 100 µl
of wash buffer only was included. The plate was incubated at 37°C for 1 h. After a wash cycle, 100 µl of goat anti-rat immunoglobulin G conjugated to HRP at a dilution of 1/1000 was added and the plate was incubated at 37°C for 1 h. After another wash cycle 100 µl of ABTS substrate solution was added to each well and the plate was incubated at room temperature for 30 min. The reaction was stopped by adding 100 µl of dH2O to each well. The absorbance of each well was read at 405 nm using a microplate reader.

2.1.4 ELISA using sera from SPF chickens

The initial experiments using serum from rats hyper-immunised with ILTV gG-GST showed that recombinant gG-His was suitable for use as an ELISA antigen. The ELISA protocol was then re-optimised for use with serum from chickens experimentally inoculated with different strains of ILTV.

2.1.4.1 Sera

Serum samples used for this assay were the same serum samples described in 2.1.1.5.

2.1.4.2 Optimisation of ELISA conditions

A series of experiments were conducted to ascertain the optimal dilution of serum for use in the gG-His antigen ELISA. A series of two-fold dilutions of a pool of 5 sera from SA2 ILTV, A20 ILTV, Serva ILTV or ΔgG ILTV vaccinated or mock vaccinated SPF chickens (commencing at 1/20 dilution) were tested. An antigen concentration of 2 µg/ml was used (determined from experiments utilising hyper-immunised rat sera ELISA) and the optimisation of this ELISA protocol then followed the same approach as described above in section 2.1.3.2. A dilution of 1/20 was selected for further use. After selecting this dilution all serum samples were then individually tested in duplicate at this dilution.
Each ELISA plate also included seven sets of standard chicken sera of known relative antibody concentration from the TropILT ELISA kit. These standard serum samples were used in duplicate at a 1/20 dilution and were included in order to monitor inter-assay variation and to enable the relative antibody concentration of the test sera to be calculated.

2.1.4.3 Calculation of antibody concentration

DeltaSoft 3 (BioMetallics Inc.) was used to determine the relative antibody concentration for each test serum from the recorded optical densities. A standard curve using the readings from the control sera was constructed and a relative antibody unit (AbU) was assigned to each standard (TropILT ELISA kit). The absorbance readings of the test samples were then plotted against the standard curve for each plate and reported as an AbU. All values were converted to a log₁₀ base and results plotted using GraphPad Prism 5 software (GraphPad Software, Inc).

Two different approaches were used to evaluate the results from this ELISA. Firstly, in order to assess the level of agreement between this ELISA and the TropILT ELISA at the level of the individual animal, the cut off value for positivity was determined based on the mean antibody concentration of the negative sera plus 1 or 2 standard deviations (STDEVs). Using these parameters contingency tables was constructed and kappa values were calculated. Secondly, the ability of this ELISA to differentiate sera from SPF birds exposed to ΔgG ILTV from those exposed to any of the other ILTV strains was tested setting a cut-off value based on the mean antibody concentration of the sera from ΔgG ILTV vaccinated group plus 1 or 2 STDEVs. The final approach was designed to assess the suitability of using this ELISA in series with TropILT ELISA at the level of the flock. This more closely represents the intended use of this ELISA in the
field, where seropositivity for ILT would be determined firstly by another method (such as ELISA using whole virus antigen) before this differential ELISA is applied to determine if seropositivity is due to exposure to ΔgG ILTV or another (gG-expressing) strain of ILTV. For this approach the cut off value was set to exclude false positive results (100% specificity).

2.1.5 ELISA using sera from commercial chickens

In order to examine the suitability of this ELISA outside of experimental conditions using SPF birds, the ELISA protocol was re-optimised for use with serum from commercial chickens.

2.1.5.1 Sera

The serum samples used for this assay were the same samples described in 2.1.1.5.

2.1.5.2 Optimisation of ELISA conditions

A series of experiments were conducted to ascertain the optimal dilution of serum from commercial chickens for use in the gG-His antigen ELISA. A series of two-fold dilutions of sera from four unvaccinated and four ILTV-vaccinated commercial chickens (commencing at 1/100 dilution) were tested. Optimisation of this ELISA protocol then followed the same approach as described above in section 2.1.3.2. A dilution of 1/400 was selected for further use and all serum samples were then individually tested in duplicate at this dilution.

Each ELISA plate also included eight sets of standard chicken sera of known relative antibody concentration. For this purpose sera from individual commercial birds vaccinated with SA2 ILTV were pooled and used as standards in two-fold dilutions in
duplicate in diluent buffer, starting from 1/100 dilutions. AbU’s were assigned to these control samples by comparing their OD values with those from the TropILT standard sera (section 2.1.4.3). These standards were included in order to monitor inter-assay variation and to enable the relative antibody concentration of the test sera to be calculated. Additional control wells were included on each plate, as described above.

2.1.5.3 Calculation of antibody concentration

DeltaSoft 3 was used to determine the AbU for each test serum as described above.
2.2 Multiplex TaqMan polymerase chain reaction assay

A TaqMan based PCR was developed in order to identify and differentiate the ΔgG ILTV vaccine virus from wild type ILTV

2.2.1 TaqMan PCR development and optimisation

2.2.1.1 Template preparation

Infectious laryngotracheitis virus DNA was extracted from the field strain CSW-1 ILTV (Bagust et al., 1986) and ΔgG ILTV (Devlin et al., 2007), both propagated in LMH cells as previously described (Devlin et al., 2006). ILTV infected cells and supernatant were harvested and the DNA was extracted using the QIAamp DNA mini kit (Qiagen) according to the manufacturer’s instruction. The DNA was eluted in 100 µl of elution buffer (10 mM Tris-Cl, 0.5 mM EDTA, pH 9.0) by centrifuging at 6000 × g for 1 min and stored at -20°C.

2.2.1.2 PCR primers and hydrolysis probes

Two sets of primers and probes were designed to detect and differentiate wild-type (WT) ILTV and ΔgG ILTV using ILTV DNA sequences. ILTV sequences in GenBank accession no. U28832 were used to design these primers and probes. For WT ILTV, the gG+ve primer pair were predicted to generate an 80 bp product. For ΔgG ILTV the ΔgG primer pair were predicted to generate a 219 bp product. The nucleotide sequence of the forward primer used for amplification of the target regions from each of WT and ΔgG ILTV was the same. The TaqMan probe for WT ILTV (gG+ve_FAM) targeted a region within the gG gene sequence. The TaqMan probe for ΔgG ILTV (ΔgG_Quaser) targeted a region within the gJ gene sequence (Figure 2.1). Primers and probes were synthesized by BioSearch Technologies (Table 2.1).
Figure 2.1 Schematic of the WT and ΔgG ILTV genome showing relative positions of primers and probes. Both forward primers target the UL47 gene. The reverse primer and probe for WT ILTV target the gG gene region whilst the primer and probe for ΔgG ILTV target the gJ gene region.

Table 2.1 Primers and probes used for ILTV TaqMan PCR assay

<table>
<thead>
<tr>
<th>Primer/Probe*</th>
<th>Orientation</th>
<th>Sequence (5’ to 3’)*</th>
<th>Target Region^</th>
</tr>
</thead>
<tbody>
<tr>
<td>gG+ve</td>
<td>Forward</td>
<td>CAGCTCGAAGTCTGAAGAGACA</td>
<td>7221-7242</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>AGCGAGCATACTAGGGAACGGT</td>
<td>7300-7278</td>
</tr>
<tr>
<td>ΔgG</td>
<td>Forward</td>
<td>CAGCTCGAAGTCTGAAGAGACA</td>
<td>7221-7242</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GCTGCACGCCAACTCCTATG</td>
<td>8317-8298</td>
</tr>
<tr>
<td>gG+ve_FAM</td>
<td></td>
<td>FAM-TGAGCGGCTTCAGTACATA GGATCGA-BHQ-1</td>
<td>7246-7272</td>
</tr>
<tr>
<td>ΔgG_Quasar</td>
<td></td>
<td>Quasar670-TGTGCAGGCTTACATAT AATTACG-BHQ-2</td>
<td>8260-8284</td>
</tr>
</tbody>
</table>

^ Gene bank accession number US28832

* BHQ: Black Hole Quencher, FAM: Fluorescein amidite, Quasar: Cyanine (Cy5) replacement
2.2.1.3 Construction of plasmids for use as qPCR standards

The 80 bp product from WT ILTV strain CSW-1 and the 219 bp product from ΔgG ILTV were amplified using conventional PCR. The 50 µl reaction mixture contained 1 mM MgCl₂, 1 × GoTaq® flexi PCR buffer (Promega), 200 µM of each dNTP, 1.25 units of GoTaq® flexi DNA polymerase (Promega), 0.2 µM of forward and reverse primer and 1 µl of extracted WT ILTV DNA or ΔgG ILTV DNA. The PCR reactions were incubated through 1 cycle of 94°C for 3 min, followed by 35 cycles of denaturation of 94°C for 30 s, annealing at 60°C for 30 s and extension at 68°C for 30 s and then one final extension cycle of 68°C for 5 min. The PCR product from each reaction was separated in a 2% agarose gel and visualised using SYBER-SAFE (Invitrogen). Bands corresponding to the correct size were identified and extracted using the QIAEX II Gel Extraction Kit according to the manufacturer’s instructions. The gel-purified PCR products were cloned into pGEM-T vector (Promega) according to the manufacturer’s instructions. The ligated products were used to transform E. coli DH5α by electroporation. Positive transformants were selected as described previously (2.1.1.2) and examined for the presence of the insert by PCR and verified by DNA sequencing using T7 and SP6 primers (Table 2.3) and BDT version 3.1 chemistry according to the manufacturer’s instruction. Plasmid DNA for each PCR product (pWT-ILTV and pΔgG-ILTV) was prepared from an overnight culture of E. coli using the Wizard plus SV miniprep kit according to the manufacturer’s instructions. The standard plasmid pWT-ILTV and pΔgG-ILTV were linearised by SalI HF according to manufacturer’s instruction and purified using QIAEX II Gel Extraction Kit. The concentration of each of the plasmid preparations (pWT-ILTV and pΔgG-ILTV) was measured using a
NanoDrop® Spectrophotometer. The copy number of the cloned ILTV DNA was calculated using the following formula:

\[
\text{Plasmid copies/µl} = \frac{\text{plasmid amount (g/µl)} \times (6.022 \times 10^{23})}{(\text{plasmid length in bp} \times 660)}
\]

2.2.1.4 Optimisation of PCR conditions

To optimise PCR conditions, different ratios of primers and probes were tested. Five ratios of gG+ve forward and reverse primer’s (1:5, 1:2, 1:1, 2:1 and 5:1) at 300 nM each were tested with the gG+ve_FAM probe at a concentration of 400 nM, 200 nM, 100 nM, 50 nM or 25 nM. Real-time PCR amplification was performed using a Stratagene Mx3000 qPCR Thermal Cycler (Agilent Technology) in a 20 µl volume containing 10 µl of 2x Brilliant II qPCR master mix and ROX reference dye (for normalisation of background) (Agilent Technology). Two microlitres of WT ILTV DNA was used as template in each reaction. Reactions were incubated for 10 min at 95°C for polymerase activation, then 40 cycles of 95°C denaturation for 30 s, 60°C annealing for 30 s and extension for 30 s at 72°C. For ΔgG ILTV, five different ΔgG ILTV primers ratios and five different ΔgG _Quasar probe concentrations were tested as described above for WT ILTV. For both PCR reactions the optimal primer ratio was found to be 1:1 and the optimal probe concentration was found to be 0.1 µM.

To estimate the efficiency of amplification using different primers and probes, a three-fold dilution series of extracted viral DNA was used as template. Amplification efficiencies and Ct values were determined using the Stratagene Mx3000 1.0 software. Following optimisation, both WT and ΔgG ILTV DNA templates were used in PCRs containing the gG+ve and ΔgG ILTV primers and the relevant probe in individual
assays to exclude non-specific amplification. A 10-fold dilution series containing $10^7 - 10^1$ and $10^6 - 10^1$ plasmid copies of pWT-ILTV and pΔgG -ILTV per 2 μl, respectively, was then used to generate standard curves for each assay and used to calculate the ILTV copy number using the Stratagene Mx3000 1.0 software.

2.2.2 Application of the TaqMan qPCRs to laboratory samples

After the initial optimisation of the TaqMan qPCR assays, DNA from eight ILTV strains, eight other poultry pathogens and chicken cellular DNA was used as template in the TaqMan qPCRs to determine the specificity of the assays.

2.2.2.1 Samples tested to determine the specificity of the TaqMan PCR

The ILTV strains used in this study were the laboratory strain CSW-1 ILTV (Bagust et al., 1986), the SA2, A20 and Serva ILTV vaccine strains, Class 8 and 9 field strains of ILTV (Blacker et al., 2011), ΔgG ILTV and a field strain of ILTV reisolated after experimental infection of chickens (bird 26 isolate) (Coppo et al., 2011).

In addition DNA or cDNA from other poultry pathogens was utilised as template, including chicken anaemia virus strain CAU269/7 (Kaffashi et al., 2008), fowl adenovirus 8b and 11 (Steer et al., 2009), avian leukosis virus (Fenton et al., 2005), M. gallisepticum S6 (Markham et al., 1992) and avian pathogenic E. coli (APEC) E3 strain (Ginns et al., 1998). DNA from LMH and CEK cells was also used.

2.2.2.2 Template preparation

All ILTV vaccine strains were propagated on the CAM of 10-day-old chicken embryos as described previously (Kirkpatrick et al., 2006). Viral DNA was extracted from homogenates of infected CAM or allantoic fluid using the QIAamp DNA mini kit.
(Qiagen) according to the manufacturer’s instruction. The field strains of ILTV were similarly propagated in chicken embryos and DNA extracted from homogenates of the infected CAM or allantoic fluid. Primary chick embryo kidney and LMH cells were grown in 6-well plates (Becton Dickinson Labware) at 37°C in a CO₂ incubator until confluent, after which cells along with culture media were harvested and 200 µl of each cell sample used to extract DNA.

Template DNA was prepared from laboratory stocks of the other poultry pathogens using the QIAamp DNA mini kit according to the manufacturer’s instruction. For preparation of template DNA from avian leukosis virus, genomic RNA was extracted using an RNeasy RNA isolation kit (Qiagen) according to manufacturer’s instructions and cDNA generated using Superscript III RNase reverse transcriptase (SSIII RT) and 200 ng of random hexamers (Invitrogen) according to the manufacturer’s instructions.

2.2.2.3 TaqMan PCR

Extracted DNA (2 µl) from the different samples was added to reactions containing 10 µl of 2 × Brilliant II qPCR master mix, 0.3 µM each primer, 0.1 µM probe, 0.375 µl of ROX reference dye and water to 20 µl. The TaqMan PCRs were performed firstly in an individual format (separate qPCRs for WT and ΔgG ILTV) using the same reaction conditions as described above (2.2.1.4). The assays were then tested in a multiplex assay that included both primer and probe sets. Each multiplex PCR reaction was carried out in a 20 µl reaction that contained 2 × Brilliant Multiplex qPCR master mix, 0.3 µM of each of the forward and reverse primers, 0.1 µM of each probe, 0.375 µl of ROX reference dye, and 2 µl of DNA template. Standard curves were generated as described above (2.2.1.4) and virus copy number and Ct values were determined and
analysed using the Stratagene Mx3000 1.0 software. All assays also contained negative control (no template control) reactions in duplicate.

2.2.3 Application of the TaqMan PCRs to clinical samples

The TaqMan PCRs were applied to clinical samples (tracheal and conjunctival swabs) in order to examine their suitability for use as a diagnostic test to detect and differentiate ∆gG ILTV and WT ILTV in infected chickens.

2.2.3.1 Clinical samples

Tracheal and ocular swabs from earlier studies (Coppo et al., 2011; Devlin et al., 2008) were utilised. The swabs were collected from SPF chickens infected with WT ILTV or vaccinated with ∆gG ILTV.

2.2.3.2 Template preparation

Tracheal swabs were placed in sterile Dulbecco’s Minimal Essential Medium (DMEM) (Sigma Aldrich) and DNA extracted from 200 µl swab sample using VX Universal Liquid Sample DNA extraction kit (Qiagen) and a Corbett X-tractor Gene Robot (QIAGEN) according to the manufacturer’s instructions. A negative control (sterile media) for each extraction was included in every column of the DNA extraction plate. A positive extraction control was also included and contained samples of WT or ∆gG ILTV grown in LMH cells. Extracted DNA was stored at -20°C until use in the TaqMan qPCR assay.

2.2.3.3 TaqMan PCR

Template DNA prepared from WT and ∆gG ILTV infected clinical samples were amplified using their respective TaqMan PCR. The clinical samples were also tested in
a multiplex assay and Ct values and virus genome copy numbers were determined and analysed as described above (2.2.2.3).

2.2.4 Application of TaqMan PCR to samples co-infected with WT and ΔgG ILTV

In order to assess the capacity of the TaqMan PCRs to detect ΔgG ILTV and WT ILTV in mixed infections, these viruses were used to experimentally co-infect LMH cells and embryonated eggs. Samples were then collected over time and the number of genome copies was determined using the TaqMan PCRs. These co-infection studies were also used to assess the growth kinetics of the WT and ΔgG ILTV under co-infection conditions.

2.2.4.1 Plaque assay

Laboratory stocks of WT and ΔgG ILTV were titrated using plaque assays. For each virus, 10 fold dilutions were prepared (10⁻¹ to 10⁻⁶) in 1 ml of sterile DMEM and 400 µl of each viral dilution added in duplicate to each well of a six well plate (Becton Dickinson Labware) containing sub-confluent LMH cells. The plates were incubated at 37°C for 1 h in a CO₂ incubator and then 2 ml of a 1% methyl cellulose overlay media was added to each well. The cells were then incubated at 37°C for three days and the plaques were counted using an inverted microscope. Plaque forming units per mL (PFU/ml) values were then calculated for each virus stock.

2.2.4.2 Co-infection of LMH cells with WT and ΔgG ILTV

LMH cells were inoculated in duplicate using a MOI of 0.002. For this purpose 2.5 × 10³ PFU of both ΔgG ILTV and WT ILTV were suspended in 200 µl of growth medium (GM) consisting of DMEM with 10% fetal bovine serum (FBS) (CSL) and 50 µg /ml of
ampicillin. The inoculum was then added to approximately $1.25 \times 10^6$ sub-confluent LMH cells in six well trays. The viruses were allowed to adsorb for one hour after which the inoculum was removed and replaced with 2 ml of fresh GM. The cells were then incubated at 37°C in a CO2 incubator. Every 24 h for 4 days the infected cells together with the supernatant were harvested and stored at -70°C for further processing. Each virus was also used separately to inoculate LMH cells using the same MOI and the cells and supernatant collected every 24 h for the following four days and stored at -70°C.

2.2.4.3 DNA template preparation

Stored infected cell cultures were thawed and DNA was extracted from 200 µl of each sample using a VX Universal Liquid Sample DNA extraction kit and a Corbett Xtractor Gene Robot as described above (2.2.3.2). Negative and positive extraction controls (distilled water and laboratory stocks of ILTV, respectively) were included in each extraction plate.

2.2.4.4 TaqMan PCR

The TaqMan PCR was conducted to determine the genome copy number of each ILTV strain in a multiplex reaction as described previously (2.2.2.3) using the same reaction conditions.

2.2.4.5 Growth kinetics

The number of viral genome copies present over time in individually infected or co-infected cell culture samples was plotted using MS Excel software.
2.2.4.6 Co-infection of embryonated eggs with WT and ΔgG ILTV

Ten day old embryonated eggs were co-infected in the allantoic cavity in triplicate with $1.0 \times 10^4$ PFU of both ΔgG ILTV and WT ILTV suspended in 100 µl of GM. Eggs were then incubated at approximately 37°C in a humidified environment and every 24 h for 5 days three eggs in each group were moved to 4°C for euthanising. Allantoic fluid was then harvested and stored at -70°C until further processing. Additional eggs were also infected individually with $1.0 \times 10^4$ PFU of either ΔgG ILTV or WT ILTV in triplicate. Allantoic fluid was collected from these eggs and stored as described above.

2.2.4.7 Template preparation

Stored allantoic fluid samples were thawed and DNA from 200 µl of each sample was extracted using VX Universal Liquid Sample DNA extraction kit and a Corbett Xtractor Gene Robot as described above. Negative and positive extraction controls were included in each extraction plate.

2.2.4.8 TaqMan PCR

The TaqMan PCR was conducted to determine the genome copy number of each ILTV strain in a multiplex reaction as described previously (2.2.2.3) using the same reaction conditions.

2.2.4.9 Growth kinetics

The number of viral genome copies present in individually infected or co-infected embryonated eggs over time was plotted against time using MS Excel software.
2.3 Generation of recombinant ILTV expressing regions of the S1 and N genes of infectious bronchitis

2.3.1 Construction of a plasmid containing IBV gene sequences

Plasmid pgGu-S1-N-gGd was constructed to contain immunogenic regions of the S1 and N genes of IBV flanked by sequences that extend 1004 bp upstream (gGu) and 989 bp downstream (gGd) of the gG gene of ILTV, respectively. The DNA from the ILTV gG flanking regions were assembled together with the DNA encoding regions of the S1 and N genes of IBV using splicing by overlap extension PCR (SOE PCR) (Horton et al., 1989, Devlin et al., 2006a). The resultant construct (gGu-S1-N-gGd) was ligated to the plasmid pGEM-T to produce pgGu-S1-N-gGd.

The DNA regions ILTVgGu and ILTVgGd were amplified by PCR using an iCycler Thermal Cycler. The 50 µl reaction mixture contained 2 mM MgSO4, 1.25 units of Platinum Taq High Fidelity DNA polymerase (Invitrogen), 1× Platinum Taq High Fidelity DNA polymerase buffer (Invitrogen), 200 µM of each dNTP, 0.2 µM of the primers 1 and 2, or 3 and 4, for the segments Gu and Gd, respectively (Table 2.2) and 2 µl of extracted ILTV DNA. The reactions were incubated through 1 cycle of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58.3°C for 30 s and extension at 68°C for 1 min and then one final extension cycle of 68°C for 5 min.

The PCR to amplify a region of the S1 gene (containing one main immunogenic epitope) and a region of the N gene from IBV utilised the same reactions components, except for primers and template. The reaction conditions were 1 cycle of 94°C for 3 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 1 min and then one final extension cycle of 72°C for 5 min. Amplification of the IBV S1 gene utilised primers 5 and 6 (Table 2.2), and
amplification of the IBV N gene utilised primers 7 and 8 (Table 2.2) and template plasmid DNA containing the S1 (nt 894-1688 from sequence U29519) and N (nt 862-1230 from sequence U52594) genes of the Vic S strain of IBV from previous studies (Chiu, 2006; Shil et al., 2011). The PCR products from each reaction were separated and visualised by agarose gel electrophoresis and amplicons of the expected size were extracted using the QIAquick Gel Extraction Kit (Qiagen) according to the manufacturer’s instructions.

Table 2.2 Primers used for construction of the rILTV-IBV

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
<th>Sequence (5’ to 3’)^</th>
<th>Product</th>
</tr>
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<tbody>
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<td>Forward</td>
<td>Gctgggctgttgctcagaga</td>
<td>ILTVgGu</td>
</tr>
<tr>
<td>2</td>
<td>Reverse</td>
<td>AAAAGTAAAAATTAGTTAACGTcatgatgtctctcagaactt</td>
<td>ILTVgGd</td>
</tr>
<tr>
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<td>Forward</td>
<td>TAGAGAGAATGAACTTTTGAccacceccagagtttttttg</td>
<td>IB-S1</td>
</tr>
<tr>
<td>4</td>
<td>Reverse</td>
<td>Ccaagaaccegctcagaac</td>
<td>IB-N</td>
</tr>
<tr>
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<td>Forward</td>
<td>aagtctgaagacacatgACGTTAACTAATTTTACTTTTTTC</td>
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</tr>
<tr>
<td>6</td>
<td>Reverse</td>
<td>TCTGGTTGAAAGTTGGGCGTTGATCCATTGGTGAGTTA</td>
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<tr>
<td>7</td>
<td>Forward</td>
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<td>8</td>
<td>Reverse</td>
<td>caaaaaacactctcgggTCAAGTTCCATTCTCCTCTA</td>
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</tr>
</tbody>
</table>

^Upper case = IBV, lower case = ILTV sequence
Figure 2.2 Generation of recombinant ILTV-IBV. (a) Schematic representation of the ILTV genome showing the unique long (UL) and unique short (US) regions. (b) Physical map showing the gene arrangement of the US region of ILTV. (c) Physical map showing the gene arrangement of the US region in an intermediate strain of ILTV expressing GFP instead of gG [ΔgG(eGFP) ILTV]. (d) Regions upstream (gGu) and downstream (gGd) of gG were amplified by PCR. (e) gGu and gGd were assembled together with the IBV S1 and IBV N gene coding sequence by SOE PCR to generate ΔgG(S1-N)ILTV by homologous recombination with ΔgG(eGFP)ILTV genomic DNA.
The four PCR products were assembled together in three stages using SOE PCR. Firstly, IB-S1 and IB-N were assembled together to produce S1-N product using a PCR mixture as described above, except with 0.2 μM of primers 5 and 8 (Table 2.2). The template DNA consisted of 2 μl of each of the extracted products from the IB-S1 and IB-N PCRs (Figure 2.2). The reactions were incubated at 94°C for 2 min, followed by 4 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 1.5 min and extension at 68°C for 3 min, then 26 cycles of denaturation at 94°C for 30 s, annealing at 64°C for 45 s and extension at 68°C for 3 min, with one final extension cycle of 68°C for 5 min. The SOE PCR products were separated and visualised by agarose gel electrophoresis and the amplicons of the expected size extracted using the QIAquick Gel Extraction Kit. Secondly, ILTV gGd was joined with the IBV S1-N DNA fragment SOE PCR using primers 4 and 5 (Table 2.2). The template DNA consisted of 2 μl of each of the extracted products of the S1-N and ILTVgGd PCRs. The SOE PCR products were extracted using the QIAquick Gel Extraction Kit and used in the final SOE PCR. The ILTV gGu was added to the S1-N-gGd DNA fragment and amplified as described above using primers 1 and 4 (Table 2.2). The PCR product (gGu-S1-N-gGd) from this final SOE PCR were extracted as described above and ligated to the plasmid pGEM-T according to the manufacturer’s instructions then used to transform E. coli DH5α by electroporation. Transformants were selected and the insert sequenced using the Big Dye Terminator (BDT) version 3.1 chemistry using primers 2, 5, 6 and 7 (Table 2.2), as well as primers T7 and SP6 (Table 2.3). An E. coli clone containing the correct DNA sequence was selected and the DNA was extracted for co-transfection experiments using the Plasmid Midi kit according to manufacturer’s instructions. Prior to co-transfection, pgGu-S1-N-gGd was linearised by digestion with the restriction
endonuclease Sa/I HF (New England Biolabs) according to the manufacturer’s instruction.

2.3.2 Co-transfection experiments

The recombinant ILTV was generated using homologous recombination which involved co-transfection of three separate DNA preparations into the nucleus of LMH cells, followed by selection and characterisation of recombinant virus. The three different DNA constructs included the plasmid described above (pgGu-S1-N-gGd, 2.3.1), ILTV genomic DNA from an intermediate strain of ILTV that expressed green fluorescent protein (GFP) instead of gG [ΔgG(eGFP)ILTV] and a plasmid expressing the ILTV ICP-4 gene (see below).

Preparation of the expression plasmid pRC-ICP4

The expression plasmid pRc-ICP4, kindly provided by Prof T. C. Mettenleiter (Federal Research Centre for Virus Diseases of Animals, Insel Riems, Germany), contained the ICP4 gene of ILTV cloned in pRc-CMV (Invitrogen) (Fuchs et al., 2000). The plasmid was electroporated into, and propagated in E. coli DH5α and extracted as described above (2.3.1).

Preparation of ILTV genomic DNA

Virions were extracted from the supernatant of ΔgG(eGFP)ILTV infected LMH cells as previously described (Devlin et al., 2006a). Virions together with detached cells were pelleted by centrifugation at 50,000 g for 1 h in a Beckman Ultrafuge, the pellet was re-suspended in 1 ml of 20 mM tris(hydroxymethyl)amino methane-HCl (pH 7.4), 1 mM ethylenediaminetetraacetic acid (EDTA) and 150 mM NaCl per original 100 cm² area of infected cells. The cells and virions were lysed by treatment with 500 µl 3% N-
lauroylsarcosinate in 75 mM Tris-HCL (pH 8.0) and 25 mM EDTA for 15 min at 65°C. After treatment with Rnase (Qiagen) 50 µg/ml for 30 min at 37°C and then Proteinase K (Invitrogen) 50 µg /ml for 2 hours at 42°C, the lysate was extracted with phenol-chloroform-isoamyl alcohol. The nucleic acid was precipitated with ethanol, pelleted and re-suspended in 10 mM Tris-HCL, 1 mM EDTA (pH 8.0). The concentration of DNA was determined using a NanoDrop®.

**Co-transfection and isolation of rILTV-IBV**

To optimise transfection conditions in LMH cells, including the ratio of transfection reagent to DNA, experiments were carried out using a plasmid expressing the GFP gene (pcD.eGFP) with the transfection reagents FuGene 6 and Fugene HD reagent (Roche) according to manufacturer’s instructions. A 5:2 ratio of reagent:DNA provided maximum transfection efficiency with Fugene HD. For co-transfection 1 µg each of ΔgG(eGFP)ILTV DNA, pRc-ICP4 and linearised pgGup-S1-N-gGd were mixed with 7.5 µl of Fugene HD and used to co-transfect sub-confluent LMH cells in a 6 well plate (Becton Dickinson). Due to the cytotoxic effects of Fugene HD, the cell medium was removed after 12 h and replaced with GM. Incubation was continued until viral plaques became visible. After 3 - 4 days of incubation, the cells and supernatant were collected, freeze-thawed and the lysate stored at -70°C.

Sub-confluent LMH cells in 6 well plates were infected with the progeny viruses and screened for expression of GFP. Transfection progeny viruses were diluted from 10 - 500 fold in GM and 200 µl was used to infect LMH cells as described above (2.2.4.2). Viral plaques that failed to express GFP were identified and selected using an inverted fluorescence microscope and a 20 µl micropipette tip.
The selected plaques were resuspended in 200 µl of GM and inoculated onto LMH cells in a 6 well plate. After 1 h incubation at 37°C the virus inoculum was removed and 1% methyl-cellulose overlay media was added. Incubation was continued until plaques had developed. Plaques were picked and inoculated onto LMH cells as described above. This process was continued until the virus had undergone three rounds of plaque purification. The virus resulting from this process was then amplified on LMH cells in 75 cm² flasks and stored at -70°C.

The recombination region of the ILTV genome of plaque-purified virus was sequenced using oligonucleotide primers from Table 2.2 and 2.3. For this, the recombination region was amplified by PCR using primers srLT-IBfwd and srLT-IBrev which were designed to anneal outside of the recombination region (Table 2.3). The amplified PCR product was purified using gel-extraction (2.3.1) and sequenced using BDT version 3.1 chemistry using primers in Table 2.2 and 2.3.

**Table 2.3 Primers used to confirm the sequence of rILTV-IBV**

<table>
<thead>
<tr>
<th>Sequencing primers</th>
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<tr>
<td>srLT-IBfwd</td>
<td>CGCGACAAGCGACTGTTAAG</td>
</tr>
<tr>
<td>srLT-IBrev</td>
<td>TCTATCCGGTGAGTTGAATC</td>
</tr>
<tr>
<td>srLT-IB1</td>
<td>CGTGGCTCCAGAGGTGTCA</td>
</tr>
<tr>
<td>srLT-IB2</td>
<td>GAAGGGCTCCATAATGACGT</td>
</tr>
<tr>
<td>srLT-IB3</td>
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</tr>
<tr>
<td>srLT-IB4</td>
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</tr>
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<td>srLT-IB5</td>
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</tr>
<tr>
<td>T7</td>
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</tr>
<tr>
<td>SP6</td>
<td>GATTTAGGTGACACTATAG</td>
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</table>
2.4 *In vitro and in ovo* characterisation of rILTV-IBV

The cell-to-cell spread characteristics, growth kinetics and gene expression profile of rILTV-IBV in LMH cells was compared to ΔgG ILTV. The growth kinetics of rILTV-IBV and ΔgG ILTV were also studied in embryonated hen eggs.

2.4.1 Cell-to-cell spread

The titre of rILTV-IBV and ΔgG ILTV stocks were determined by plaque assay as described above (2.2.4.1). To assess the cell-to-cell spread characteristics of the viruses the mean area of plaques induced by rILTV-IBV and ΔgG ILTV in LMH cells were calculated 48, 72 and 96 h after infection with $2.5 \times 10^3$ PFU of virus. At each time point, photomicrographs of 13 – 16 well isolated plaques of each virus were captured and saved for analysis. The circumference of each plaque was measured using ImageJ software and used to calculate the plaque areas. The results were statistically compared between the two viruses using Student’s *t*-test.

2.4.2 Growth kinetics

To compare the growth kinetics of rILTV-IBV and ΔgG ILTV *in vitro* and *in ovo*, growth curves were determined in both LMH cells and 10 day old embryonated eggs, respectively. Approximately $2.5 \times 10^3$ PFU of rILTV-IBV or ΔgG ILTV in 200 µl of GM was used to inoculate approximately $1.25 \times 10^6$ sub-confluent LMHs in a 6-well plate in duplicate (MOI of 0.002). The supernatant was replaced after 1 h with fresh GM and incubation at 37°C and 5% CO₂ was continued. The cells and supernatant from wells of infected LMH cells (n = 2) were harvested every 24 h for 5 days and stored at -70°C. The collected samples were titrated on LMH cells using a plaque assay (2.2.4.1).
To study the growth kinetics of the viruses in embryonated eggs, 1.0 × 10^4 PFU of rILTV-IBV or ΔgG ILTV in 100 µl of GM was inoculated into the allantoic cavity of 10 day old embryonated SPF eggs in triplicate. Eggs were then incubated at approximately 37°C in a humidified environment and every 24 h for 5 days three eggs in each group were moved to 4°C to euthanise the embryo. Allantoic fluid was then harvested and stored at -70°C until further processing. ILTV genome copy numbers in the collected samples were determined by qPCR targeting the UL15 gene of ILTV (Mahmoudian et al., 2011). The VX Universal Liquid Sample DNA extraction kit and a Corbett X-tractor Gene Robot were used to extract viral DNA from 200 µl of allantoic fluid as described above (2.2.3.2). Extracted DNA was stored at -20°C until use in the UL15 ILTV qPCR.

2.4.3 Gene transcription studies

To examine the transcription of the IBV S1-N gene fragment from rILTV-IBV during infection of LMH cells, a pair of primers were designed to target the S1-N gene fragment (Table 2.4) and used to quantitate IBV S1-N mRNA levels using qPCR. Primers targeting the ILTV UL47 gene were used to measure ILTV UL47 mRNA levels. Primers targeting the chicken gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were used to assess mRNA levels of this cellular housekeeping gene (Table 2.4).

RNA was prepared from LMH cells infected in triplicate with 2.5 × 10^4 of either rILTV-IBV or ΔgG ILTV, or mock-infected with sterile GM. At 1, 3 and 6 h after infection, the cells in three wells in each group were washed with cold PBS and RNA was harvested using the RNeasy RNA isolation kit according to manufacturer’s instructions. The purity and concentration of the extracted RNA were determined using
NanoDrop®. Two micrograms of extracted RNA was digested with 2 U of amplification grade DNase I at 37°C for 30 min according to manufacturer’s instruction. Following heat inactivation of the reaction at 65°C for 10 min, 1 µg of RNA was used to generate cDNA using 200 U Superscript III RNase reverse transcriptase, 200 ng of random hexamers (Invitrogen) in a volume of 20 µl and 4× reaction buffers according to the manufacturer’s instructions. An RT control reaction that contained dH₂O instead of enzyme was included.

Table 2.4 Primers used for gene expression studies

<table>
<thead>
<tr>
<th>Primer</th>
<th>Orientation</th>
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<td>tranSN</td>
<td>Forward</td>
<td>CGTAATGAAACAGGTTCTCAGGCTATTGAGAACCAG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GGGTCATCCCTTGACCACAGTATGAAACTC</td>
</tr>
<tr>
<td>UL 47</td>
<td>Forward</td>
<td>GATACAGCCGTTCAATGCC</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>ATGCCCTACTTTCAACGC</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Forward</td>
<td>CACTATCTTCCAGGAGCGTG</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>CAGATGAGCCCCAGCCTTC</td>
</tr>
</tbody>
</table>

Amplification of cDNA was performed in duplicate for each triplicate sample at each time point using Stratagene Mx3000 qPCR Thermal Cycler. Each 20 µl qPCR reaction contained 10 µl of Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen), 0.25 µl of 10 µM each primer and 2 µl of cDNA template. Reactions were incubated for 2 min at 50°C, then 5 min at 95°C, followed by 45 cycles of 60°C for 30 s and 95°C for 30 s followed by a dissociation curve. A no RT control for each sample and each primer set was included in each experiment together with a no template control (NTC). For each sample the threshold value was automatically determined using the Stratagene Mx3000 1.0 software.
2.4.4 Protein expression studies

Western blots and immunofluorescence assays were performed to examine the expression of foreign IBV protein from rILTV-IBV in infected LMH cell cultures.

**Western blot**

Sub-confluent LMH cells in six well trays were washed twice with PBS before inoculation in duplicate with $2.5 \times 10^4$ PFU of either rILTV-IBV or ΔgG ILTV in 200 μl of serum free DMEM. Cells were incubated for 1 h at 37°C in a CO₂ incubator before the inoculum was removed and replaced with 1 ml of fresh serum free medium. The cells were incubated overnight and the presence of visible plaques confirmed the following day using light microscopy. Infected cells and supernatant were harvested and the cells pelleted by centrifugation. Cell pellets and supernatants were incubated at 100°C for 5 min in 20 μl of 2 × SDS-PAGE sample buffer. Western blotting was performed as described in section 2.1.1.2, and probed with rabbit anti-GST-S1 polyclonal serum at a dilution of 1/1000 (Chiu, 2006) and detected with goat anti-rabbit immunoglobulin G conjugated to HRP (GE healthcare).

**IFA**

Sub-confluent LMH cells were grown in Lab-Tek II chamber slide systems (Nalge Nunc International) and were inoculated with either rILTV-IBV, ΔgG-ILTV or sterile media as described in section 2.2.4.2, except 10-fold serial dilutions of each virus was used. After 1 day of incubation the presence of viral plaques were confirmed using light microscopy and the cells were washed twice with PBS before fixing for 5 minutes at room temperature with 90% (v/v) methanol. Fixed cells were washed once with PBS, and probed with either monoclonal antibodies to ILTV (TropBio) (York and Fahey,
or rabbit anti-GST-S1 polyclonal serum (Chiu, 2006) diluted 1:100 in 1% bovine serum albumin in PBS-T (100 µl for 40 minutes at room temperature with gentle shaking). Slides were washed twice with PBS-T before adding either rabbit anti-mouse FITC-conjugated anti-mouse antibody (DAKO) or goat anti-rabbit FITC-conjugated monoclonal antibody (DAKO) as appropriate in a 1/250 dilution in 1% bovine serum albumin in PBS-T (100 µl). After 40 minutes incubation at room temperature with gentle shaking the slides were washed twice with PBS-T, mounted with fluorescence mounting medium (DAKO) and visualised on a fluorescent microscope.
2.5 *In vivo* characterisation of rILTV-IBV

2.5.1 Safety and vaccine efficacy of rILTV-IBV administered by eye-drop

The safety and immunogenicity of rILTV-IBV was first investigated using eye-drop vaccination of 21 day old SPF chickens as described previously (Devlin et al., 2007) and subsequent challenge with IBV 28 days after vaccination. A total of 80 birds were randomly assigned to three groups. Groups 1 and 2 each contained 25 birds whilst group 3 contained 30 birds. Birds in group 1 were mock-vaccinated and mock-challenged, birds in group 2 were mock-vaccinated and challenged, and birds in group 3 were vaccinated and challenged. The experimental design is summarised in Table 2.5. Ethics approval for this experiment was granted by the Animal Ethics Committee, The Faculty of Veterinary Science, The University of Melbourne (AEC No. 1112144.1). Each group of birds was held in a separate isolator and provided irradiated feed and water *ad libitum* throughout the experiment.

Safety of rILTV-IBV following vaccination via eye-drop

At the start of the experiment serum samples were collected from each bird and all birds were weighed. Birds in group 3 were then inoculated by eye-drop with 30 µl of GM containing $3.0 \times 10^3$ PFU of rILTV-IBV. Birds in groups 1 and 2 were mock vaccinated with 30 µl of GM only.

After vaccination, all birds were monitored for clinical signs of respiratory disease. Four days after vaccination serum was collected from five randomly selected birds from groups 1 and 2, and ten birds randomly selected from group 3. These birds were euthanised by exposure to halothane, weighed and their weight gains since the time of vaccination were calculated and compared between groups using a Student’s *t*-test. The
lumen of the trachea was examined for gross pathological lesions. A scoring system was used to grade the severity of the lesions based on the amount of tracheal mucous exudate: 0, no exudate; 1, small amount of exudate; 2, moderate amount of exudate; 3, large amount of exudate; and 4, very large amount of exudate. A transverse section of proximal trachea was collected and preserved in Bouin’s fixative. These sections were processed and stained with Haematoxylin and Eosin (H&E) prior to examination using light microscopy. Tracheal histopathological lesions were scored on a scale of 0 - 5 as previously described (Guy et al., 1990). Gross pathology and histopathology results were compared between groups using the Mann-Whitney test.

Conjuctival swabs and tracheal scrapings were collected during post-mortem examination and stored in viral transport medium and processed for viral DNA extraction using the VX Universal Liquid Sample DNA extraction kit and a Corbett Xtractor Gene Robot as described previously. Extracted DNA was used to quantitate rILTV-IBV using the UL15 qPCR as described previously (2.4.2).

Vaccine efficacy of rILTV-IBV following vaccination via eye-drop

Four weeks after vaccination all remaining birds were weighed and serum was collected from each bird. Birds in groups 2 and 3 were then challenged with IBV Vic-S (Poulvac® Bron Vic-S, Zoetis Australia) by eye-drop using 50 × the vaccine dose recommended by the manufacturer. Birds in group 1 were mock-challenged with sterile vaccine diluent. Birds were monitored for clinical signs of disease for 5 days following challenge. Ten birds were culled by exposure to halothane at two different time-points (3 and 5 days after challenge) and subjected to post-mortem examinations. At these two
time-points, birds from each experimental group were randomly selected and a serum sample was collected before euthanasia.

The birds were weighed and their weight gain since the time of challenge were calculated and compared between groups using a Student’s t-test.

Gross tracheal lesions were scored as described above and a transverse section of proximal trachea was collected into Bouin’s fixative, processed and stained with H&E. The stained sections were examined using light microscopy and scored on the basis of five different histological parameters: sloughing of epithelium, epithelial hyperplasia/metaplasia, inflammation, loss of mucus glands and loss of cilia. All parameters were scored using a scale of 0 to 3, with a score of 0 corresponding to no pathology; a score of 1 to mild pathology; a score of 2 to moderate pathology and a score of 3 to severe pathology. Histopathological scores were compared between groups using a Mann-Whitney test. In addition to these parameters the thickness of the tracheal mucosa was assessed by measuring the mucosa at 10 different sites around the tracheal lumen using an inverted microscope. Average tracheal thicknesses in µm were compared between groups using a Student’s t-test.

Tracheal scrapings were collected and placed in viral transport medium for the detection of IBV RNA and ILTV DNA using PCR. Viral DNA or RNA was extracted as described above (2.2.3.2) using a Corbett X-tractor Gene Robot. The UL15 qPCR was used to detect the recombinant vaccine virus by qPCR as described above (2.4.2). For detection of challenge virus, IBV RNA was amplified using a Superscript III platinum SYBR green one-step qRT-PCR kit (Invitrogen). All qPCR reactions were conducted in
a 25 µl reaction. The qPCR to detect IBV RNA utilised the All1-F and Del1-R primers which amplify a 433 bp product in the 3´ UTR region of IBV (Hewson et al., 2009).
Table 2.5 Experimental design to assess the safety and vaccine efficacy of rILTV-IBV delivered via eye drop

<table>
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<th>Experimental Group</th>
<th>No. of birds (21 days old)</th>
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<th>Day 31</th>
<th>Day 33</th>
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<td></td>
<td>Vaccination</td>
<td>Post Mortem</td>
<td>Challenge</td>
<td>Post Mortem</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(Eye drop)</td>
<td>(Eye drop)</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>1</td>
<td>25</td>
<td>Mock</td>
<td>5 birds</td>
<td>Mock</td>
<td>10 birds</td>
<td>10 birds</td>
</tr>
<tr>
<td>2</td>
<td>25</td>
<td>Mock</td>
<td>5 birds</td>
<td>IBV Vic-S</td>
<td>10 birds</td>
<td>10 birds</td>
</tr>
<tr>
<td>3</td>
<td>30</td>
<td>rILTV-IBV</td>
<td>10 birds</td>
<td>IBV Vic-S</td>
<td>10 birds</td>
<td>10 birds</td>
</tr>
</tbody>
</table>
Detection of antibodies against IBV and ILTV in chicken sera using ELISA and haemagglutination inhibition assay

Serum samples collected 4 and 28 days after vaccination with rILTV-IBV, and 3 and 5 days after challenge with IBV, were analysed for the presence of antibodies against IBV using IDEXX IBV ELISA (IDEXX laboratories) according to the manufacturer’s instructions. Serum samples collected before challenge were analysed for the presence of antibodies against ILTV using the Trop-ILT ELISA kit (Tropbio) according to the manufacturer’s instructions. A haemagglutination inhibition (HI) assay was also conducted to test for the presence of anti-IBV antibodies in serum samples collected 4 and 28 days after vaccination and 5 days after challenge. The assay was conducted as described previously (King and Hopkins, 1983).

Detection of antibodies against IBV S1 in chicken sera using Western blotting

To prepare protein for use in Western blotting, recombinant IBV GST-S1 protein was produced and purified from *E. coli* JM109 cultures containing the pGEX-4T-1 expression vector (Amersham Biosciences) expressing S1-GST (Chiu, 2006). Purified GST protein (without a fusion protein) was also used in this study (a kind gift from Nadeeka Wawegama, The University of Melbourne). The purity of the GST-S1 and GST proteins was determined on SDS-PAGE gel stained with Coomassie blue, and also on Western blots stained with an anti-GST antibody (GE Healthcare) (diluted 1/2000 in PBS-T) or rabbit anti-GST-S1 polyclonal serum (Chiu, 2006) (diluted 1/1000 in PBS-T) as described above (2.1.1.2).

To detect antibodies against IBV S1 in chicken sera, a pool of sera collected from chickens vaccinated with rILTV-IBV 28 days after vaccination (diluted 1/100 in PBS-
T) was used to probe PVDF membranes containing transferred purified GST-S1. The pooled serum was simultaneously tested against purified GST protein only. Sera collected 28 days after vaccination from each bird in group 3 were tested individually in western blot to GST-S1 as described in section 2.1.1.2. Bound chicken antibody was detected using rabbit anti-chicken immunoglobulin G conjugated to HRP (GE healthcare) used at a dilution of 1/1000 in PBS-T.

2.5.2 Safety and vaccine efficacy of rILTV-IBV administered by the intra-tracheal route

An additional study of the safety and vaccine efficacy of rILTV-IBV was conducted using the intra-tracheal route of vaccination. This experiment utilised 30 SPF birds at 21 days of age. The birds were randomly assigned to three groups of 10 birds. Birds in group 1 were mock-vaccinated and mock-challenged. Birds in group 2 were mock-vaccinated and challenged with IBV. Birds in group 3 were vaccinated with rILTV-IBV and challenged with IBV. Each group of birds was held in a separate isolator and provided irradiated feed and water ad libitum. The experimental design is summarised in Table 2.6. Ethics approval for this project was granted by the Animal Ethics Committee, The Faculty of Veterinary Science, The University of Melbourne (AEC No. 1312878.1).

Safety of rILTV-IBV following intra-tracheal vaccination

At the start of the experiment, each bird was weighed and a serum sample collected. Birds in group 3 were vaccinated by the intra-tracheal route with 300 µl of GM containing $3.0 \times 10^3$ PFU of rILTV-IBV. All birds in groups 1 and 2 were mock vaccinated with 300 µl of GM only. Birds were monitored for clinical signs of
respiratory disease for 15 days following vaccination and results were compared between groups using a Mann-Whitney test. Intra-tracheal and conjunctival swabs were collected 2, 4 and 6 days after vaccination from all live birds in order to investigate the replication of rILTV-IBV *in vivo*. These swabs were collected into viral transport media and stored at -70 °C before viral DNA was extracted and used in qPCR targeting the ILTV UL15 gene, as described above.

Sixteen days after vaccination all birds were weighed and weight gains since the time of vaccination were calculated and compared between groups using a Student’s *t*-test.

**Vaccine efficacy of rILTV-IBV following intra-tracheal vaccination**

Sixteen days after vaccination serum samples were collected from each bird and then all birds in groups 2 and 3 were challenged with IBV Vic-S by eye-drop as described in section 2.5.1. Birds in group 1 were mock challenged with sterile diluent only. Birds were then monitored for clinical signs of disease for 5 days following challenge. Five days after challenge serum samples were collected from all remaining birds and all birds were euthanised by exposure to halothane, weighed and subjected to post-mortem examination.

At post-mortem, gross tracheal pathology was scored and tissue samples were collected and examined as described in section 2.5.1. This included collection of a section of proximal trachea for histopathological analysis and collection of tracheal mucosal scrapings to examine for the presence of IBV RNA and ILTV DNA using PCR.

ELISAs and Western blot were used to detect antibodies against ILTV and IBV in the serum samples collected 16 days after vaccination and 5 days after challenge, as described in section 2.5.1.
Table 2.6 Experimental design to assess the safety and vaccine efficacy of rILTV-IBV delivered by intra-tracheal inoculation

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>No. of birds (21 days old)</th>
<th>Day 0</th>
<th>Day 2, 4 and 6</th>
<th>Day 16</th>
<th>Day 21</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Vaccination (Intra-tracheal)</td>
<td>Tracheal and conjunctival swabbing</td>
<td>Challenge (Eye drop)</td>
<td>Post Mortem</td>
</tr>
<tr>
<td>1</td>
<td>10</td>
<td>Mock</td>
<td>All birds</td>
<td>Mock</td>
<td>10 birds</td>
</tr>
<tr>
<td>2</td>
<td>10</td>
<td>Mock</td>
<td>All birds</td>
<td>IBV</td>
<td>10 birds</td>
</tr>
<tr>
<td>3</td>
<td>10</td>
<td>rILTV-IBV</td>
<td>All birds</td>
<td>IBV</td>
<td>10 birds</td>
</tr>
</tbody>
</table>
3 Development of an enzyme linked immunosorbent assay

3.1 Introduction

This chapter describes the development of a differential ILTV ELISA. Generally whole virus has been used as antigen in ELISAs to detect serum antibodies against ILTV (Meulemans and Halen, 1982). However such ELISAs are not suitable for serologically differentiating between birds infected with gene-deletion mutants of ILTV from those infected with a wild-type ILTV. The aim of this study was to develop a differential ELISA to use as a companion diagnostic tool in conjunction with the ΔgG ILTV vaccine. Two different recombinant ILTV gG proteins, expressed and purified in two different expression systems, were assessed for their suitability for use as ELISA antigen.

3.2 Expression and purification of recombinant gG

3.2.1 Expression and purification of opt gG-MBP in E. coli

The nucleotide sequence of the gG cloned in the expression vector pMALp4x was found to be identical to the codon optimised sequence. A single E. coli Rosetta Gami colony containing the gG-pMALp4x (opt_gG pMALp4x) was selected and used for expression and purification of the recombinant gG. Following purification, a protein of approximately 76 kDa was visualised following staining with Coomassie brilliant blue, although a number of smaller proteins were also observed (Figure 3.1A). The recombinant protein was detected by both polyclonal anti-gG rat sera and pooled A20 ILTV vaccinated chicken sera (Figure 3.1B and C). Purified MBP was observed to be approximately 42 kDa in size in a Coomassie brilliant blue stained SDS-PAGE gel (Figure 3.1A). The polyclonal anti-gG rat sera did not react with the tag protein MBP.
(Figure 3.1B), but pooled A20 ILTV vaccinated chicken sera did react with the MBP tag protein (Figure 3.1C).

3.2.2 Expression and purification of gG-His in insect cells

Another form of full length ILTV gG was purified as a gG-His fusion protein using recombinant baculovirus expression and a Ni-NTA agarose purification system. For these experiments, protein was purified from both the supernatant and cell fractions of baculovirus infected Sf9 cells. Coomassie brilliant blue staining of purified proteins

Figure 3.1 Expression, purification and detection of recombinant opt gG-MBP and MBP purified from E. coli cultures. (A) Coomassie brilliant blue stained polyacrylamide gel of uninduced (lane 1) and induced (lane 2) culture of E. coli JM 109 cells harboring pMAL-p4X/optgG and of purified opt gG-MBP (lane 3) and MBP (lane 4), (B) Western blot of opt gG-MBP (lane 1) and MBP (lane 2) probed with rat antiserum to ILTV gG, (C) Western blot of opt gG-MBP (lane 1) and MBP (lane 2) probed with pooled sera from chickens vaccinated with A20 ILTV
showed two bands of similar sizes at around 42 kDa (Figure 3.2A). The same pattern was observed when probed with polyclonal anti-gG rat sera and pooled A20 ILTV vaccinated SPF chicken sera (Figure 3.2B and C).

![Figure 3.2 Detection of recombinant gG-His purified from infected insect cells. (A) Purified gG-His following SDS-PAGE and staining with Coomassie brilliant blue, (B) Western blot of gG-His stained with rat antiserum to ILTV gG, (C) Western blot of gG-His stained with pooled sera from chickens vaccinated with A20 ILTV](image)

3.3 Development of ILTV gG ELISA

3.3.1 ELISA optimisation using gG-MBP as antigen

A series of two-fold dilutions of gG-MBP ranging in concentration from 0.25 µg/ml to 8 µg/ml was tested with pooled sera from A20 ILTV vaccinated SPF chickens. A concentration of 2 µg/ml of the antigen was determined as optimum using the checkerboard titration method (Figure 3.3A).
Pooled sera from SPF birds vaccinated with A20 ILTV, as well as pooled sera from field vaccinated birds, were then tested to determine the optimum serum dilution for use in ELISA. For both sets of pooled serum, similar absorbance values were obtained against the gG fusion protein compared to MBP alone (Figure 3.3B and C). These results were consistent with those obtained in Western blotting which showed possible nonspecific reactions between the tag protein MBP and chicken sera (Figure 3.1C).

As a result of these nonspecific reactions, the gG-MBP antigen was not considered further for developing a recombinant gG ILTV ELISA.

### 3.3.2 ELISA optimisation using gG-His as antigen

Optimisation of the concentration of gG-His antigen and dilution of serum in a checkerboard titration assay using serial dilutions of polyclonal anti-gG rat sera showed that the optimum concentration of antigen was 2 µg/ml. In the same assay, negative control serum (rat antisera against an irrelevant protein: 3CT of Equine rhinitis B virus) did not show any significant binding to the gG-His (Figure 3.4A).
Figure 3.3 Optimisation of ELISA conditions using opt gG-MBP antigen. (A) Dilutions of pooled sera from SPF chickens vaccinated with A20 ILTV were tested against different concentrations of gG-MBP antigen. An antigen concentration of 2 µg/ml was selected for further use, (B) There was no apparent difference in the absorbance values recorded for the fusion protein gG-MBP compared to the MBP tag on its own using pooled sera from SPF chickens vaccinated with A20 ILTV (C) There were no apparent difference in the absorbance values recorded for the fusion protein gG-MBP compared to the MBP tag on its own using pooled sera from commercial chicken vaccinated with SA2 ILTV
Figure 3.4 Optimisation of ELISA conditions using gG-His antigen. (A) Serial dilutions of rat antiserum to ILTV gG and to an irrelevant protein were tested against different concentrations of antigen, 0.25, 0.5, 2 and 6 µg/ml. An antigen concentration of 2 µg/ml was selected for further use, (B) Dilutions of a pooled sera from experimental SPF birds inoculated with ∆gG, SA2, A20 or Serva ILTV, or mock inoculated with sterile media were tested. A dilution of 1:20 was determined to be optimal for test sera from SPF birds, (C) Sera from four unvaccinated and four ILTV-vaccinated commercial birds were tested. A dilution of 1:400 was determined to be optimal for test sera from commercial birds.
Different dilutions of sera from SPF chickens experimentally vaccinated with A20, SA2 or Serva ILTV vaccines, as well as dilutions of sera from commercial chickens vaccinated with SA2 ILTV, were tested in the gG-His ELISA. A dilution of 1/20 was determined to be optimal for sera from SPF chickens (Figure 3.4B). A dilution of 1/400 was determined to be optimal for sera from commercial chickens (Figure 3.4C).

3.3.3 gG-His ELISA testing of field sera

Serum samples collected from the commercial birds were tested individually in duplicate using the optimised ELISA conditions. The relative antibody concentrations were calculated by plotting the absorbance value of test sera on the standard curve generated using a serum assigned arbitrary antibody units. The geometric mean of relative antibody concentration was different between vaccinated and unvaccinated birds (Figure 3.5). Cut off values for positivity were calculated based on the geometric mean relative antibody concentration of the sera from the unvaccinated birds plus 1 or 2 STDEVs and used to determine the agreement level with the results from Trop-ILT commercial ELISA. The cut-off value of geometric mean plus 1 STDEV produced gG-His ELISA results that had the highest level of agreement (‘very good’) with the results from the commercial ELISA (Table 3.1). The cut-off value was also used to calculate the sensitivity and specificity of the assay (Table 3.1).
Figure 3.5 ELISA testing of field sera from vaccinated and unvaccinated commercial birds. Chicken sera were used at 1:400 dilution against purified recombinant ILTV gG-His. Bars indicate the geometric mean values for each group. The geometric mean titres for vaccinated (2.159) and unvaccinated (1.171) groups were found to be significantly different with $(P < 0.001)$.

Table 3.1 Contingency table showing agreement between the gG-His and Trop-ILT ELISAs using sera from ILTV-vaccinated and unvaccinated commercial birds. A cut-off value for the gG-His ELISA of 1.50 (mean ELISA value of the negative sera plus 1 STDEV) was used.

<table>
<thead>
<tr>
<th></th>
<th>gG-His ELISA</th>
<th>Trop-ILT ELISA*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Positive</td>
<td>40</td>
<td>6</td>
</tr>
<tr>
<td>Negative</td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>46</strong></td>
<td><strong>30</strong></td>
</tr>
</tbody>
</table>

* Agreement = Very Good (Kappa value = 0.821)

^ Sensitivity = 1.000, Specificity = 0.800
3.3.4 gG-His ELISA testing of experimental SPF chicken sera

Each serum sample from experimentally infected SPF birds was tested in duplicate (Figure 3.6). The results from this ELISA were analysed using three methods. Firstly, the results from this ELISA were compared with the commercial Trop-ILT ELISA to determine an agreement value. In this case, cut-off values for positivity were calculated based on the geometric mean relative antibody concentration of the sera from the unvaccinated birds plus 1 or 2 STDEVs. Sera from birds vaccinated with ΔgG ILTV were excluded from this analysis. The cut-off value of geometric mean plus 1 STDEV produced gG-His ELISA results that had the highest level of agreement (‘moderate’) with the results from the commercial ELISA (Table 3.2).

Secondly, to differentiate sera from SPF birds vaccinated with ΔgG ILTV from those vaccinated with any other ILTV strains, ELISA cut-off values were determined based on the geometric mean antibody concentration of the sera from ΔgG ILTV vaccinated group plus 1 or 2 STDEVs. Using these cut-off values, a contingency table was constructed to determine sensitivity and specificity of gG-His ELISA and the results were tabulated (Table 3.3).
Figure 3.6 ELISA testing of sera from experimental SPF birds. Chicken sera were used at 1:20 dilution against purified recombinant ILTV gG-His. Sera were grouped based on the strain of ILTV used for vaccination. Sera from unvaccinated SPF birds (unvac) are grouped together. Bars indicate the geometric mean values for each group. The geometric mean titres of the A20 (1.582) and Serva ILTV groups (1.585) were found to be significantly different (P < 0.05) to that of the ∆gG ILTV group, but not to that of the SA2 ILTV group. The titres in the SA2, A20 and Serva ILTV all were found to be significantly different to the titres in the unvaccinated group (P < 0.05).
Table 3.2 Contingency table showing agreement between the gG-His and Trop-ILT ELISAs using sera from ILTV-vaccinated and unvaccinated SPF birds. A cut-off value for the gG-His ELISA of 1.35 (mean ELISA value in sera from unvaccinated birds plus 1 STDEV) was used.

| gG-His ELISA*|^ | Positive | Negative | Total |
|---------------|----------|----------|--------|
| Trop-ILT ELISA* | Positive | 45 | 14 | 59 |
|               | Negative | 3 | 16 | 19 |
|               | Total | 48 | 30 | 78 |

* Agreement = Moderate (Kappa value = 0.506)

[^] Sensitivity = 0.763, Specificity = 0.842

Table 3.3 Contingency table for calculating the sensitivity and specificity of the gG-His ELISA in SPF birds exposed to ΔgG or other strains of ILTV using cut-off values of 1.57 and 1.70 (mean ELISA value in sera from ΔgG ILTV vaccinated birds plus 1 and 2 STDEVs, respectively)

<table>
<thead>
<tr>
<th>ILTV exposure</th>
<th>gG-His ELISA cut-off = 1.57^</th>
<th>gG-His ELISA cut-off = 1.70*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
</tr>
<tr>
<td>ΔgG</td>
<td>3</td>
<td>15</td>
</tr>
<tr>
<td>Other</td>
<td>30</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>33</td>
<td>44</td>
</tr>
</tbody>
</table>

[^] Sensitivity = 0.508, Specificity = 0.833

* Sensitivity = 0.271, Specificity = 1.000

Finally, the gG-His ELISA was tested for its ability to differentiate birds infected with ΔgG ILTV from those infected with any other strains of ILTV at a group or flock level
rather than at the level of individual birds. This is expected to represent more-precisely the intended use of this assay in the field. First seropositivity against ILT in any particular flock would be determined by a method such as ELISA using whole virus antigen before applying this differential ELISA to determine if seropositivity is due to exposure to ΔgG ILTV or any other gG-expressing strains of ILTV. For this purpose, the cut-off value was set at lowest level to exclude all false positive results in the ΔgG ILTV inoculated group which is a cut-off of 1.70 resulting in 100% specificity (Table 3.3). Then the percentage of the positive birds in other ILTV inoculated groups was calculated to be 27.11%. This value of 27.11% was used to calculate a preliminary probability based sampling strategy to use in the field. For a flock of any size in which serum antibodies against ILTV had been detected using Trop-ILT ELISA, a total of eleven serum sample that tested positive using the TropILT ELISA would need to be tested further using the ILTV gG-His ELISA to be more than 95% confidence of detecting a positive sample in flocks exposed to a gG-expressing strain of ILTV (Cannon and Roe, 1982). Using this approach one or more positive test results using the gG-His ELISA would indicate the flock has been exposed to a gG-expressing strain of the ILTV. The absence of a positive test result would indicate the flock had only been exposed to ΔgG ILTV.

3.3.5 Reproducibility of the gG-His ELISA

The standards from three separate assays using sera from commercial birds vaccinated with SA2 were plotted to examine inter-assay reproducibility (Figure 3.7). Similarly, the standards from seven assays used to test sera from SPF chickens were also examined (Figure 3.8). Both assays showed minimal inter-assay variability.
Figure 3.7 Reproducibility of the gG-His ELISA for testing chicken sera from commercial chickens. Sera from SA2 vaccinated commercial birds were pooled and used as standards. The absorbance values from the standards used in three assays were plotted against the reciprocal of the serum dilution. Minimal inter-assay variability was detected.

Figure 3.8 Reproducibility of the gG-His ELISA for testing sera from SPF chickens. Standard chicken sera with known relative antibody concentration (from TropILT ELISA kit) were used as standards. The absorbance values from the standards used in seven assays were plotted against the titre of the serum standards. Minimal inter-assay variation was detected.
3.4 Discussion

An ELISA was developed to detect serum antibodies against ILTV gG. Two recombinant proteins, one produced by bacteria (*E. coli*) and the other by baculovirus were tested for their suitability for use as antigen in the ELISA. Purification of codon optimized gG-MBP from an *E. coli* expression system produced protein of the anticipated size following SDS-PAGE electrophoresis, as well as smaller bands that were likely to be either breakdown products or premature termination of the desired protein. Protein produced in this system was found to have an unacceptable level of non-specific reactivity between the MBP tag and the chicken sera. Non-specific binding to MBP has also been reported using sera from other species (Marcipar et al., 2004).

Recombinant gG-His produced using the baculovirus expression system was found to be more suitable for use as an ELISA antigen. Baculovirus expressed antigen has been used successfully in ELISA studies previously (Breslin et al., 2001; Martinez-Torrecuadrada et al., 2000; Zoth et al., 2011). Unlike bacterial expression systems that lack post-translational glycosylation processes (Chang et al., 2002), the intracellular modification of proteins in insect cells is similar to those in eukaryotic cells, thus resulting in protein folding and three-dimensional protein structures more similar to those obtained from eukaryotic cells (Miller, 1988). For these reasons, baculovirus expression can be a superior method for producing antigens requiring glycosylation for use in ELISA and other applications (Sanchez-Martinez et al., 1991; Verschoor et al., 1993; Wan et al., 1995). Previous studies have shown that baculovirus expressed ILTV gG-His maintains its function as a chemokine binding protein *in vitro* (Devlin et al., 2010). In this study two similar sized bands were detected in the purified preparation of gG-His, this is most likely due to differences in processed forms of ILTV gG-His,
resulting in the presence of both forms in the combined cell and supernatant fractions of the insect cell cultures.

The ELISA developed using recombinant gG-His was able to differentiate between sera from ILTV-vaccinated commercial birds and unvaccinated commercial birds. When results from this ELISA were compared to the results from a commercial ELISA (Trop-ILT) there was a very good level of agreement between the two tests (kappa value of 0.821). These results show that ILTV gG is immunogenic during natural infection with ILTV and are consistent with results from an earlier study investigating this viral glycoprotein (Kongsuwan et al., 1993). These results suggest that this ELISA could be used successfully in the field to differentiate birds exposed to ILTV from birds that have not been exposed to ILTV. Only birds exposed to SA-2 ILTV were tested, however as different ILTV strains are considered to be antigenically homogenous (Bauer et al., 1999), it is expected that this ELISA would be similarly useful for detecting serum antibodies against ILTV gG following infection of commercial chickens with another vaccine or field strains of virus.

When this ELISA was used to test sera from SPF birds the level of agreement between the results from this ELISA and those from the Trop-ILT ELISA was only moderate (kappa value of 0.506). The reasons for the poorer performance of this ELISA using sera from SPF birds is unclear but could be related to a different level or type of immune response induced in the SPF chickens compared to the commercial chickens. This could be due to the different genetic backgrounds of the chickens (Bumstead, 1998; Chang et al., 2011; Zhang et al., 2006), the different environmental conditions, or the different infection histories of the birds, though the infection history of the commercial birds was not known. It is interesting to note that the optimised ELISA
conditions required a much lower dilution of sera from SPF birds (1:20 dilution) than from commercial birds (1:400) suggesting that there was a much lower concentration of serum antibodies against ILTV gG in vaccinated SPF birds compared with vaccinated commercial birds. As all the sera collected from vaccinated SPF birds tested positive using the Trop-ILT ELISA this suggests that the vaccinated birds did produce a serum antibody response to ILTV but may not have produced a strong serum antibody response to gG specifically.

The ability of this ELISA to discriminate between SPF birds infected with ΔgG ILTV and SPF birds infected with other ILTV strains such as A20, SA2 or Serva ILTV was tested in this study. The sensitivity and specificity values of the assay were calculated based on the known exposure status of the birds. These values describe the performance of the ELISA at the level of the individual animal. However, if this ELISA were to be used as a companion diagnostic tool with the ΔgG ILTV vaccine in the field, then the ELISA may be more useful when applied at the level of the flock instead. In this present study a different antibody profile was demonstrated in the group of birds exposed to ΔgG ILTV, compared to birds exposed to the commercial ILTV vaccines. It is possible that these differences at the flock-level could be exploited in disease control or eradication programs. One preliminary probability-based sampling strategy to achieve this at field level was outlined in this study as an example. The ΔgG candidate vaccine has only ever been used under experimental conditions in SPF birds, so currently a more comprehensive evaluation of this ELISA as a companion diagnostic tool is limited. Future studies to assess the performance of this ELISA in commercial chickens under field conditions would allow the usefulness of this ELISA to discriminate between flocks of birds infected with ΔgG ILTV and those infected with other ILTV strains.
4 Development of a TaqMan real-time polymerase chain reaction assay

4.1 Introduction

In acute forms of ILT, infection can spread rapidly and mortality rates can reach up to 50%. In the early stages of an outbreak it can be helpful to use molecular diagnostic tools to detect the presence of the virus, as serum antibody to ILTV may not yet be detectable. Molecular diagnostic tools such as PCR have been developed to enhance viral detection. Some of these PCR techniques have been coupled with RFLP to detect and simultaneously characterise different strains of ILTV (Kirkpatrick et al., 2006). Real-time PCR has a number of advantages over conventional PCR assays, including enhanced sensitivity, rapidity and the ability to quantify the number of virus present in a sample (Creelan et al., 2006; Crespo et al., 2007; Mahmoudian et al., 2011). Real-time qPCR assays utilising fluorogenic hydrolysis probes (TaqMan PCR assays) have been used to improve the specificity of qPCR assays (Callison et al., 2007a). Importantly, the use of real-time qPCR to quantify and differentiate vaccine virus from WT infection would be beneficial in a disease control program. This chapter describes the development of TaqMan real-time PCR for the detection, differentiation and quantitation of ΔgG and WT (gG+ve) strains of ILTV.

4.2 Assay development and validation

4.2.1 Assay optimisation

The sequence of the gG+ve and ΔgG ILTV primers and hydrolysis probes (Table 4.1) were compared to sequences in the Genbank database using BLASTn®. The only matching sequences were from the targeted regions of the ILTV genome. Each gG+ve and ΔgG differential TaqMan PCR assay was optimised using different primer ratios
and probe concentrations. A forward and reverse primer ratio of 1:1 for each primer pair with a final concentration of 0.3 µM for each primer, and a final probe concentration of 0.1 µM, were found to be optimal and produced the highest efficiency of amplification.

The efficiency of amplification was first estimated by performing the individual assays using a three-fold dilution series of both WT and ΔgG ILTV genomic DNA. The standard curve generated using WT genomic DNA in the gG+ve TaqMan qPCR assay showed an efficiency of amplification of 90.0% with a coefficient of determination (R²) of 0.996. The standard curve generated using ΔgG ILTV genomic DNA in the ΔgG TaqMan qPCR assay showed an efficiency of amplification of 98.1% and a R² of 0.965. There was no amplification of template ΔgG ILTV DNA using the gG+ve TaqMan assay. Similarly, there was no amplification of template WT ILTV DNA using the ΔgG TaqMan assay.

The standard curves generated using serial ten-fold dilution of the plasmids pWT-ILTV or pΔgG-ILTV indicated that the individual assays had average efficiencies of 97.85% (ranging from 93.9% to 105.1%, n = 6) and 99.6% (ranging from 92.0% to 106.0%, n = 6), respectively. Average R² values of 0.998 (ranging from 0.996 to 1.000, n = 6) and 0.995 (ranging from 0.992 to 0.999, n = 6), respectively, were recorded.

The detection limit for each assay was determined to be 10 genome copies. This was the highest dilution of plasmid that consistently generated a Ct value that was lower than the Ct values produced from any negative PCR control samples.

4.2.2 Assay specificity

DNA extracted from different strains of ILTV, chicken cells, or other chicken pathogens was used as template in the individual gG+ve and ΔgG TaqMan qPCR assays. No
amplification was observed in either assay when DNA was extracted from CEK or LMH cells and used as template. Similarly, no amplification was observed when DNA from other avian pathogens (chicken anaemia virus, fowl adenovirus, avian leukosis virus, *Mycoplasma gallisepticum*, avian pathogenic *E. coli*) was used as template. The gG+ve TaqMan qPCR assay successfully amplified DNA from different gG+ve ILTV strains, whilst the ΔgG TaqMan qPCR assay only amplified DNA from ΔgG ILTV (Table 4.1). Viral genome copy numbers present in the test samples were calculated using the standard curves generated from 10-fold serial dilutions of plasmid. When the same samples were tested by the TaMan qPCRs in a multiplex format the results were consistent with those obtained from each individual assay (Table 4.1).

4.2.3 Application to clinical samples

The Taqman qPCR assays were applied to clinical samples in order to assess the suitability of each assay to serve as diagnostic tool to detect and differentiate the ΔgG candidate vaccine strain of ILTV from other ILTV strains in clinical specimens. DNA extracted from tracheal and conjunctival swabs collected from experimentally infected chickens was used as template in the individual and multiplex TaqMan PCR assays. The gG+ve TaqMan qPCR assay amplified WT ILTV DNA from all clinical samples expected to contain WT ILTV but not from those expected to contain ΔgG ILTV. Similarly the ΔgG TaqMan qPCR amplified ΔgG ILTV DNA from all clinical samples expected to contain ΔgG ILTV but not from those expected to contain WT ILTV (Table 4.1). Viral genome copy numbers present in the clinical samples were calculated using the standard curves generated from 10-fold serial dilutions of plasmid. When the TaqMan qPCRs were tested in a multiplex format the results were consistent with those from the individual assays (Table 4.1).
4.2.4 Assay reproducibility

Assay reproducibility was assessed by determining the coefficient of variation (CV) between independent assays performed using 10-fold serial dilutions of pWT-ILTV or pΔgG-ILTV. When the mean Ct values of standards were compared from 6 independent assays low inter-assay coefficients of variation were detected in the gG+ve ILTV qPCR assay (0.021 - 0.042) and in the ΔgG ILTV qPCR assay (0.014 - 0.039) (Tables 4.2 and 4.3). An average inter-assay standard deviation of 0.65 (0.53 - 1.01) and 0.61 (0.23 - 0.93) was obtained for standard curves from pWT-ILTV and pΔgG-ILTV respectively (Table 4.2 and 4.3).
Table 4.1 Detection and differentiation of ILTV in laboratory and clinical samples using TaqMan qPCR

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>ILTV strain</th>
<th>Individual TaqMan assays Ct (copy number&lt;sup&gt;▲&lt;/sup&gt;)</th>
<th>Multiplex Taqman assay Ct (copy number&lt;sup&gt;▲&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>WT ILTV</td>
<td>ΔgG ILTV</td>
</tr>
<tr>
<td>Laboratory samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>A20</td>
<td>24.39 (26420)</td>
<td>NT</td>
</tr>
<tr>
<td>2</td>
<td>SA2</td>
<td>25.53 (12550)</td>
<td>NT</td>
</tr>
<tr>
<td>3</td>
<td>Serva</td>
<td>23.55 (45720)</td>
<td>NT</td>
</tr>
<tr>
<td>4</td>
<td>Class 7</td>
<td>25.20 (15560)</td>
<td>NT</td>
</tr>
<tr>
<td>5</td>
<td>Class 8</td>
<td>22.92 (69160)</td>
<td>NT</td>
</tr>
<tr>
<td>6</td>
<td>Class 9</td>
<td>27.74 (2987)</td>
<td>NT</td>
</tr>
<tr>
<td>7</td>
<td>CSW 1</td>
<td>22.15 (113600)</td>
<td>NT</td>
</tr>
<tr>
<td>8</td>
<td>ΔgG</td>
<td>NT</td>
<td>19.71 (147600)</td>
</tr>
<tr>
<td>9</td>
<td>ΔgG</td>
<td>NT</td>
<td>19.09 (244900)</td>
</tr>
<tr>
<td>Clinical samples</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6711T</td>
<td>CSW 1</td>
<td>33.22 (84)</td>
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</tr>
<tr>
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<td>NT</td>
</tr>
<tr>
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<td>CSW 1</td>
<td>36.06 (13)</td>
<td>NT</td>
</tr>
<tr>
<td>6719T</td>
<td>CSW 1</td>
<td>26.69 (5926)</td>
<td>NT</td>
</tr>
<tr>
<td>6720T</td>
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<td>26.55 (6489)</td>
<td>NT</td>
</tr>
<tr>
<td>6720E</td>
<td>CSW 1</td>
<td>30.65 (447)</td>
<td>NT</td>
</tr>
<tr>
<td>6723T</td>
<td>CSW 1</td>
<td>28.38 (1966)</td>
<td>NT</td>
</tr>
<tr>
<td>6724T</td>
<td>CSW 1</td>
<td>34.21 (44)</td>
<td>NT</td>
</tr>
<tr>
<td>2341E</td>
<td>ΔgG</td>
<td>NT</td>
<td>30.69 (53)</td>
</tr>
<tr>
<td>2342E</td>
<td>ΔgG</td>
<td>NT</td>
<td>28.29 (300)</td>
</tr>
<tr>
<td>2343E</td>
<td>ΔgG</td>
<td>NT</td>
<td>31.35 (33)</td>
</tr>
<tr>
<td>2345E</td>
<td>ΔgG</td>
<td>NT</td>
<td>31.39 (32)</td>
</tr>
<tr>
<td>2342T</td>
<td>ΔgG</td>
<td>NT</td>
<td>29.71 (107)</td>
</tr>
<tr>
<td>2344T</td>
<td>ΔgG</td>
<td>NT</td>
<td>31.23 (36)</td>
</tr>
</tbody>
</table>

<sup>▲</sup> 2 µl template DNA used, NT-not tested, ND-not detected
### Table 4.2 Inter-assay variations in Ct values obtained using serial dilutions of pWT-ILTV in the gG+ve TaqMan PCR

<table>
<thead>
<tr>
<th>log_{10} (copy number) of plasmid</th>
<th>Assay</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>7</td>
<td>13.06</td>
<td>12.87</td>
<td>13.69</td>
<td>14.29</td>
</tr>
<tr>
<td>6</td>
<td>17.00</td>
<td>16.04</td>
<td>16.64</td>
<td>17.68</td>
</tr>
<tr>
<td>5</td>
<td>20.41</td>
<td>19.13</td>
<td>20.60</td>
<td>20.88</td>
</tr>
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<td>4</td>
<td>23.77</td>
<td>22.70</td>
<td>23.94</td>
<td>24.04</td>
</tr>
<tr>
<td>3</td>
<td>26.95</td>
<td>26.11</td>
<td>27.43</td>
<td>27.44</td>
</tr>
<tr>
<td>2</td>
<td>30.50</td>
<td>29.31</td>
<td>31.19</td>
<td>30.96</td>
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<tr>
<td>1</td>
<td>34.15</td>
<td>32.19</td>
<td>34.55</td>
<td>34.53</td>
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</table>

### Table 4.3 Inter-assay variations in Ct values obtained using serial dilutions of pΔgG-ILTV in the ΔgG TaqMan PCR

<table>
<thead>
<tr>
<th>log_{10} (copy number) of plasmid</th>
<th>Assay</th>
<th>Mean</th>
<th>SD</th>
<th>CV</th>
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<td></td>
<td>1</td>
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<td>33.19</td>
<td>32.38</td>
<td>33.83</td>
<td>32.68</td>
</tr>
</tbody>
</table>


4.2.5 Application to samples co-infected with WT and ΔgG ILTV

The ILTV TaqMan assay, in a multiplex format, was applied to samples collected from cell cultures and embryonated hen eggs that were either separately infected or co-infected with WT and ΔgG ILTV. The multiplex assay successfully detected both viruses present in the co-infected samples. The growth kinetics of the two viruses was compared to each other under individual infection and co-infection condition (Figure 4.1 and 4.2). Under co-infection and individual infection conditions in LMH cells the number of viral genome copies of WT ILTV was higher than the number of viral genome copies of ΔgG ILTV. Under co-infection and individual infection conditions in embryonated hen eggs the number of viral genome copies of WT ILTV was also higher than the number of viral genome copies of ΔgG ILTV.
Figure 4.1 Growth curves of WT and ΔgG ILTV in LMH cells. LMH cells were co-infected in duplicate with WT and ΔgG ILTV using a MOI of 0.002 for each virus (A), or were infected in duplicate separately with WT or ΔgG ILTV using the same MOI (B). Viral genome copy numbers were calculated using the multiplex ILTV TaqMan qPCR and the mean value was plotted against days post infection.
Figure 4.2 Growth curves of WT and ΔgG ILTV in embryonated eggs. Ten-day-old embryonated SPF eggs were co-infected in triplicate with WT and ΔgG ILTV using a MOI of 0.002 for each virus (A), or were infected in triplicate separately with WT or ΔgG ILTV using the same MOI (B). Viral genome copy numbers were calculated using the multiplex ILTV TaqMan qPCR and the mean value and standard deviation was plotted against days post infection.
4.3 Discussion

A TaqMan qPCR assay was developed to detect and differentiate the ΔgG ILTV vaccine strain of ILTV from ILTV strains that contain the gG gene. The assays were used individually and in a multiplex format to detect, differentiate and quantitate ILTV DNA in laboratory and clinical samples. The assays were also applied to viral cultures taken at several time points after inoculation to investigate the growth kinetics of ΔgG and WT ILTV in cultured cells and embryonated eggs under single and co-infection conditions.

The standard curves produced using 10-fold dilutions of pWT-ILTV or pΔgG-ILTV maintained linearity for seven and six dilution steps respectively, with high average efficiencies of amplification (97.85% and 99.6%, respectively) and low coefficients of variation (0.998 and 0.995, respectively). There were no differences between the standard curves generated using the individual assay or multiplex assay formats.

Validation of the gG+ve and ΔgG TaqMan assays using dilutions of viral genomic DNA showed that the assays were efficient in amplifying DNA from the targeted virus but did not amplify DNA from the non-target virus. These assays compare favorably to a previously published TaqMan qPCR assay utilising a probe targeting the gC gene of ILTV. The standard curve for that assay maintained linearity for at least five dilution steps with an overall efficiency of 94.54% and R² of 0.994 (Callison et al., 2007a). The lower detection limit of the gC-based TaqMan assay in the previous study was determined to be 25 viral template copies (Callison et al., 2007a), while the detection limit of the TaqMan assays in this present study was 10 viral template copies.

The specificity of the TaqMan qPCR assays were demonstrated by their ability to selectively amplify DNA from the targeted ILTV strain(s), including under co-infection
conditions. Importantly the gG+ve TaqMan PCR assay was able to amplify DNA from all ILTV strains tested, except the ΔgG ILTV strain. This is important if these assays were to be used in the field to detect and differentiate the ΔgG ILTV vaccine from other strains of ILTV, as previous studies have shown that a large number of different field strains (including strains derived from conventionally attenuated vaccines) are typically present in poultry industries (Bagust and Johnson, 1995; Blacker et al., 2011; Kirkpatrick et al., 2006). All of these field strains of ILTV would be expected to contain the gG gene and hence be detectable by the gG+ve TaqMan PCR assay. Neither of the TaqMan assays amplified chicken DNA or DNA from other avian pathogens that are likely to be present in clinical samples.

During an outbreak of disease in the field, DNA based techniques to detect ILTV could be applied immediately, before serum antibody is detectable, which is typically around two weeks after infection (Crespo et al., 2007; Guy and Garcia, 2008; Sander and Thayer, 1997). The detection and differentiation of ILTV using TaqMan PCR has a number of advantages over viral isolation, and even over PCR-RFLP, including the rapidity of detection compared to isolating and growing virus in cell culture or eggs (Callison et al., 2007a; Williams et al., 1992c). Restriction fragment analysis of viral genomic DNA has been used for over a decade to assist epidemiological studies (Guy et al., 1989). More recently PCR-RFLP of individual genes has been used for the detection and differentiation of ILTV isolates (Kirkpatrick et al., 2006). Restriction fragment analysis of genomic DNA requires extraction and purification of viral genomic DNA, which requires isolation and propagation of the virus, is expensive and time consuming. PCR-RFLP analysis of individual PCR products requires amplification of large regions of individual genes capable of differentiating between strains (Kirkpatrick et al., 2006).
It is also time consuming, requires relatively high quality of viral DNA (to amplify large PCR products) and relatively expensive. In a recent study in our laboratories, a real-time SYBR Green qPCR assay targeting ILTV UL 15 was found to be more sensitive than the conventional PCRs that it was tested against, whilst several conventional PCRs produced negative results for specimens that were positive in the SYBR Green qPCR (Mahmoudian et al., 2011)

Following ILTV infection, ILTV can generally be detected in the conjunctiva and trachea for 1-14 days (Guy et al., 1992; Guy and Garcia, 2008; Hughes and Jones, 1988; Williams et al., 1992c) and it is in these early stages of disease where PCR based methods of detection are most useful. Under field conditions when ILTV is circulating in a large flock, the staggered nature of infection of individual birds, as well as potential reactivation of infection, means that there is likely to be an extended timeframe (beyond the 6-8 day period of shedding in an individual infected bird) when ILTV can be detected by PCR. The low copy numbers of virus detected in some of the clinical samples tested in this study may be related to the sampling time, with reduction in virus occurring over time (Callison et al., 2007a).

The TaqMan PCR assays, in a multiplex format, were applied to examine the growth kinetics of the WT and ΔgG strains of ILTV in cultured LMH cells and embryonated hen eggs. Previously, the replication of these viruses in cultured cells had been examined under single-infection conditions using PFU assay, with no differences in replication kinetics being observed (Devlin et al., 2006a). The development of the TaqMan PCR assays allowed the replication of these viruses to be examined under co-infection conditions.
In cultured cells WT ILTV had consistently higher copy numbers compared to ΔgG ILTV, including at the first time point immediately after co-infection with equal quantities of each virus, as determined by PFU assay. TaqMan PCR assays detect both infectious and non-infectious virus, in contrast to PFU assays that detect only infectious virus. The results therefore suggest that WT ILTV produces more non-infectious virus particles compared to ΔgG ILTV. This may be related to better adaptation of ΔgG ILTV to growth in cultured cells, which is consistent with the passage history of ΔgG ILTV (between 10-15 more passages in cultured cells compared to WT ILTV). Despite the consistently higher copy numbers of WT ILTV causing an upwards shift of the WT ILTV growth curve, the slopes of the growth curves for WT ILTV and ΔgG ILTV in LMH cells were similar, indicating comparable replication kinetics. In embryonated hen eggs there was a high degree of variation in copy numbers of WT and ΔgG ILTV at each time point but a similar trend to that observed in cultured cells was apparent.

In summary, the ILTV TaqMan qPCR assays described in this chapter could detect and differentiate ΔgG ILTV from WT ILTV, and from other strains of ILTV that contain the gG gene. These assays were highly specific, highly sensitive and highly reproducible and could be used in individual or multiplex formats. They were applied successfully to laboratory samples and were used to examine the replication kinetics of ΔgG and WT ILTV under co-infection conditions. Their successful application to clinical samples shows their potential to be used in the field. These TaqMan PCR assays, along with the ΔgG ILTV vaccine and the ELISA to detect serum antibodies to ILTV gG (Shil et al., 2012), represents a suite of tools that could be used in a DIVA-based approach to the control or eradication of ILT.
5 The generation and initial characterisation of recombinant ILTV expressing regions of the S1 and N genes of infectious bronchitis virus

5.1 Introduction

This chapter describes the development of a recombinant gG-deficient strain of ILTV expressing regions of the S1 and N genes of infectious bronchitis virus. Vectored vaccines for the control of infectious bronchitis have previously been developed using adenovirus and fowl pox virus as vectors (Johnson et al., 2003; Wang et al., 2009). Previous studies have also demonstrated that ILTV can be successfully used as a vector to deliver antigens from avian influenza (Pavlova et al., 2009). Vectored vaccines that are capable of inducing immunity to multiple different pathogens have a number of potential advantages over traditional vaccines, including reduced costs to poultry producers arising from administration of fewer vaccines to their flocks. In the experiments described in this chapter, regions of the S1 and N genes of IBV were inserted into a previously developed strain of ∆gG ILTV expressing GFP [∆gG(eGFP)ILTV] (Devlin et al., 2007) using the process of homologous recombination. The resulting recombinant virus (rILTV-IBV) was characterised in vitro and in ovo.

5.2 Generation of recombinant ILTV-IBV

Following co-transfection of LMM cells with ILTV pgGu-S1-N-gGd, ∆gG(eGFP)ILTV and plasmid pRC-ICP4 (section 2.3.2) the progeny rILTV-IBV virus was detected by observing plaques that did not express GFP (Figure 5.1). Following three rounds of plaque purification under overlay medium the recombination region of the progeny virus was PCR amplified and the DNA sequence was determined. Sequence analysis
showed that the IBV S1 and N gene construct successfully replaced the eGFP gene in the genome of ∆gG(eGFP)ILTV.

Figure 5.1 Photomicrograph of the LMH cell monolayer after co-transfection. The absence of GFP expression (centre plaque) was used to differentiate recombinant virus (rILTV-IBV) from the parent virus [ΔgG(eGFP)ILTV] (magnification 10 x).

5.3 *In vitro and in ovo* characterisation of rILTV-IBV

5.3.1 Cell to cell spread

The sizes of the viral plaques induced by rILTV-IBV and ΔgG ILTV infection of LMH cell cultures are summarised in the Table 5.1. The mean area of the plaques (n = 15-16) resulting from rILTV-IBV infection of LMH cells was not significantly different from that of the plaques (n = 13-15) induced by ΔgG ILTV at any of the time points.
Table 5.1 The size of plaques induced by rILTV and ΔgG ILTV in LMH cell cultures

<table>
<thead>
<tr>
<th>Virus</th>
<th>Plaque area in mm² (mean ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>48 hpi* (n)</td>
</tr>
<tr>
<td>rILTV-IBV</td>
<td>0.83 ± 0.07 (16)a</td>
</tr>
<tr>
<td>ΔgG ILTV</td>
<td>0.96 ± 0.10 (14)a</td>
</tr>
</tbody>
</table>

*Values with the same superscript letter in the same column were not significantly different

* hpi = hours post infection

5.3.2 Growth kinetics

There was no apparent difference in the growth kinetics of rILTV-IBV in comparison to ΔgG ILTV in LMH cells (Figure 5.2). There was no significant difference in the growth kinetics of these two viruses when inoculated into the allantoic cavity of embryonated eggs (Figure 5.3).
Figure 5.2 Growth kinetics of rILTV-IBV and ΔgG ILTV in LMH cells. Cultures of LMH cells were infected with 2500 PFU of ΔgG ILTV or rILTV-IBV in duplicate. Cells and supernatants were harvested every day for five days and the virus present in the collected samples was quantitated using a PFU assay in LMH cells. Mean viral titres are shown.
Figure 5.3 Growth kinetics of rILTV-IBV and ΔgG ILTV in embryonated eggs. The allantoic cavity of embryonated hen eggs at 10 days of incubation were inoculated with $10^3$ PFU of ΔgG ILTV or rILTV-IBV in triplicate. Allantoic fluid was collected every day for five days and the virus present in the collected samples was quantitated using qPCR. The mean and standard deviation of viral genomes detected at each time point are shown.

5.3.3 Transcription of IBV S1-N in cell cultures infected with rILTV-IBV

The expression of IBV S1-N by rILTV-IBV during infection of LMH cells was assessed by measuring mRNA abundance in RT-qPCR using primers targeting the IBV S1-N recombination region of rILTV-IBV. Relative abundance of mRNA for IBV S1-N, ILTV UL47 and the housekeeping gene GAPDH, in rILTV-IBV and/or ΔgG ILTV infected LMH cells were found as summarised in Table 5.2. There was an increase in the relative abundance of IBV S1-N mRNA at 1, 3 and 6 h post infection of rILTV-IBV in LMH cells. IBV S1-N mRNA was not detected in cells infected with ΔgG ILTV.
Table 5.2 Relative abundance of mRNA for IBV S1-N, ILTV UL47 and GAPDH in LMH cells infected with rILTV-IBV or ΔgG ILTV

<table>
<thead>
<tr>
<th>Hours post infection</th>
<th>IBV S1-N (rILTV-IBV)</th>
<th>ΔgG ILTV</th>
<th>ILTV UL47 (rILTV-IBV)</th>
<th>ΔgG ILTV</th>
<th>GAPDH (rILTV-IBV)</th>
<th>ΔgG ILTV</th>
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<tr>
<td>1</td>
<td>31.76 ± 0.23a</td>
<td>no Ct</td>
<td>29.60 ± 0.16a</td>
<td>30.19 ± 0.14a</td>
<td>13.85 ± 0.03a</td>
<td>13.80 ± 0.67a</td>
</tr>
<tr>
<td>3</td>
<td>26.33 ± 0.74b</td>
<td>no Ct</td>
<td>25.48 ± 0.21b</td>
<td>25.64 ± 0.20b</td>
<td>14.01 ± 0.46a</td>
<td>14.00 ± 0.29a</td>
</tr>
<tr>
<td>6</td>
<td>22.73 ± 0.66c</td>
<td>no Ct</td>
<td>22.38 ± 0.10c</td>
<td>22.55 ± 0.31c</td>
<td>14.00 ± 0.14a</td>
<td>13.88 ± 0.52a</td>
</tr>
</tbody>
</table>

a, b, c Values with the same superscript letter at each time point in each column were not significantly different.

5.3.4 Detection of IBV S1-N protein in cell cultures infected with rILTV-IBV

Western blotting and IFA techniques were used in an attempt to detect IBV S1 protein expression in rILTV-IBV infected LMH cells. Rabbit anti-GST-S1 polyclonal serum used in Western blotting did not detect IBV S1 protein in either the cell or supernatant of rILTV-IBV infected LMH cells (results not shown). The same serum used in IFA did not produce a fluorescence signal in rILTV-IBV infected LMH cell cultures. Monoclonal antibodies against ILTV produced a strong fluorescence signal when applied to rILTV-IBV and ΔgG-ILTV infected cells using the same IFA technique (results not shown).
5.4 Discussion

A recombinant strain of ILTV containing an IBV S1-N gene fragment in place of the ILTV gG gene was generated and characterised in vitro and in ovo. The S1-N gene fragment of IBV that was inserted into the ILTV genome was derived from the Vic S strain of IBV. This is a serotype B vaccine strain of IBV, as determined by virus neutralisation assay, with immunogenic properties (Chiu, 2006). The region of the S1 glycoprotein used to generate rILTV-IBV is immunogenic and is conserved in all Australian IBV isolates (Ignjatovic and Sapats, 2005). The S1 glycoprotein has been identified as a major inducer of protective immune responses (Cavanagh et al., 1986a; Song et al., 1998b). Previous studies in our laboratories have used this same S1 IBV gene fragment in a M. gallisepticum expression vector which was subsequently shown to be successful in partially reducing pathological changes induced by IBV challenge (Shil et al., 2011).

To generate rILTV-IBV an intermediate strain of ILTV expressing GFP instead of gG [AgG(eGFP)ILTV] (Devlin et al., 2007) was utilised in co-transfection experiments that resulted in the removal of the GFP reporter gene and its replacement with the IBV S1 and N gene fragment via the process of homologous recombination. Similar approaches to generating ILTV recombinants have been used in other ILTV studies (Fuchs et al., 2007; Fuchs et al., 2000; Luschow et al., 2001). The detection of non-fluorescent virus plaques (indicating a successful homologous recombination event) in transfection progeny from such experiments can be difficult (Pavlova et al., 2009), however in the absence of an ILTV bacterial artificial chromosome (BAC) to facilitate the manipulation of the ILTV genome, this process of homologous recombination remains necessary. Sequence analysis of the recombination region of plaque-purified rILTV-
IBV revealed a successful in frame sequence insertion of IBV S1 and N gene fragment in the gG gene deleted area of the ILTV genome.

The rILTV-IBV displayed *in-vitro* growth characteristics similar to those of ΔgG-ILTV. There was no significant difference in the size of the viral plaques induced by rILTV-IBV and ΔgG-ILTV in LMH cells. Similarly the growth kinetics of rILTV-IBV and ΔgG-ILTV were comparable in both LMH cells and embryonated hen eggs. These results indicate that the presence of the IBV S1-N gene fragment in the ILTV genome did not alter the ability of the virus to replicate or to spread from cell-to-cell. Most commercial ILTV vaccines are propagated in SPF eggs (Guy et al., 1991) and therefore the unaltered *in ovo* growth kinetics of the rILTV-IBV is an advantage for propagation of rILTV-IBV on a scale required for a potential commercial vaccine in future.

In these studies the presence of IBV S1 protein in rILTV-IBV infected cells could not be detected using Western-blot or IFA. However mRNA transcripts of the IBV S1-N gene were detected by RT-qPCR and the transcripts were shown to increase in relative abundance over time. These findings are similar to those from a previous study examining the expression the same IBV S1 gene fragment in *M. gallisepticum* transformants (Chiu, 2006). In the *M. gallisepticum* studies the presence of IBV S1 protein could not be detected with polyclonal rabbit sera against GST-SI of IBV using Western blotting techniques, however Northern dot blot hybridisation showed the presence of mRNA transcription in some of the transformants. The study suggested that real-time RT-qPCR may be a more sensitive method to detect S1 expression.

Other studies of recombinant IBV vaccine candidates have also favoured the detection of IBV mRNA transcripts over the detection of IBV protein. Expression of the complete
S1 gene in the recombinant fowl adenovirus vaccine was confirmed by RT-PCR at 20 hours post-infection of cell cultures (Johnson et al., 2003) but the detection of S1 protein was not reported. In a different study on a recombinant ILTV vaccine candidate expressing H5 haemagglutinin (HA) and N1 neuraminidase (NA) of highly pathogenic avian influenza (HPAIV), the expression of the HPAIV proteins were enhanced by inserting immediate-early human cytomegalovirus (HCMV-IE) promoters along with synthetic introns in the 5’ non-translated region of the avian influenza virus HA and NA genes. Using this system HPAIV proteins were able to be detected in infected cell cultures and the enhanced protein expression may have helped to improve the protection induced by the vaccine against H5N1 challenge (Pavlova et al., 2009). Similar approaches to expressing IBV proteins using ILTV could be employed in future studies if required.

The studies described in this chapter have characterised the rILTV-IBV candidate vaccine in vitro and in ovo. Importantly in vivo studies in chickens are required to determine if the expression of the IBV S1-N gene fragment by rILTV-IBV is sufficient to induce an immune response following vaccination. In vivo studies are also required to examine the safety of the rILTV-IBV. Safety is an important consideration in vaccine development and although the safety of the parent ΔgG-ILTV vaccine candidate has been demonstrated (Devlin et al., 2007; Devlin et al., 2006a), the safety of rILTV-IBV must also be assessed.
6 Safety and vaccine efficacy of rILTV-IBV in SPF chickens

6.1 Introduction

This chapter describes the \textit{in vivo} characterisation of rILTV-IBV in order to determine if the recombinant virus may be suitable for use as a potential vaccine to protect birds against challenge with IBV. Although there are many parameters that need to be examined for any candidate vaccine, two fundamental parameters are vaccine safety and vaccine efficacy. The studies in this chapter aimed to provide a preliminary investigation on the safety and efficacy of rILTV-IBV after delivery via eye-drop or intra-tracheal inoculation. The two routes of delivery were studied as vaccine safety and efficacy are known to vary according to the route of vaccine administration (Coppo et al., 2013; Devlin et al., 2008). For ILTV vaccines, delivery via eye-drop is considered to be safer than delivery via intra-tracheal inoculation, but potentially less immunogenic (Devlin et al., 2008; Devlin et al., 2007). In order to examine the vaccine efficacy of the rILTV-IBV, an IBV challenge protocol utilizing an overdose of the Vic S vaccine strain of IBV was also developed in this study.

6.2 Vaccine safety and efficacy following delivery via eye-drop

6.2.1 Safety of rILTV-IBV

\textbf{Clinical signs}

No clinical signs associated with ILTV infection were observed in any of the vaccinated or mock-vaccinated birds over the four days following vaccination via eye-drop, although a number of birds in group 3 (vaccinated with rILTV-IBV) developed vent lesions due to pecking. One bird died in group 3 14 days after vaccination but this was not found to be related to ILTV infection.
Percentage weight gain

The percentage weight gain of the birds in the three different groups over the 4 days following vaccination are summarised in Table 6.1. The vaccinated birds (group 3) showed a mean percentage weight gain (14.64%) that was significantly lower than the mean percentage weight gain of the mock-vaccinated birds in group 1 (31.75%, \( p < 0.001 \)) and group 2 (27.58%, \( p < 0.001 \)).

The percentage weight gain of the birds in the three different groups over the 28 days following vaccination are summarised in Table 6.2. The weights of female and male birds were analysed separately at this time point. There was no significant difference between the mean percentage weight gain of female and male birds in each of the three groups.

Table 6.1 Percentage weight gain 4 days after eye-drop vaccination

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>n</th>
<th>Weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± STDEV</td>
</tr>
<tr>
<td>1</td>
<td>Mock</td>
<td>5</td>
<td>31.75 ± 3.20\textsuperscript{a}</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>5</td>
<td>27.58 ± 2.40\textsuperscript{a}</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>10</td>
<td>14.64 ± 4.19\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a,b} Values with the same superscript letter in the same column were not significantly different (Student’s t-test)
Table 6.2 Percentage weight gain 28 days after eye-drop vaccination

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Weight gain (%)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n</td>
<td>Female</td>
<td>Mean ± STDEV</td>
<td>n</td>
</tr>
<tr>
<td>1</td>
<td>Mock</td>
<td>11</td>
<td>283.9 ± 24.50&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>314.5 ± 18.87&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>9</td>
<td>297.2 ± 31.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11</td>
<td>330.8 ± 24.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>8</td>
<td>301.7 ± 3 9.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11</td>
<td>319.3 ± 32.83&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values with the same superscript letter in the same column were not significantly different (Student’s *t*-test)

Tracheal pathology

The tracheal gross pathology and histopathology results obtained during post-mortem examinations 4 days after vaccination are summarised in Table 6.3. There was no significant difference in the tracheal gross pathology and histopathology scores between the three groups.

Replication of recombinant ILTV-IBV

To investigate the replication of rILTV-IBV following vaccination, DNA extracted from the conjunctival and tracheal scrapings collected 4 days after vaccination was used as template in a qPCR targeting the UL15 gene of ILTV. Viral DNA was not detected in any of the tested samples.
Table 6.3 Tracheal gross and microscopic pathology scores 4 days after vaccination with rILTV-IBV

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>n</th>
<th>Gross pathology Median (range)</th>
<th>Histopathology Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>5</td>
<td>0 (0-1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-0)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>5</td>
<td>0 (0-0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-0)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>10</td>
<td>0 (0-2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-1)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values with the same superscript letter in the same column were not significantly different (Mann-Whitney test)

**Antibody detection**

Sera collected from birds 4 and 28 days after vaccination were tested for antibodies against ILTV and IBV using ELISA. Antibodies were not detected in any of the tested serum samples. Serum samples were also tested for antibodies against IBV using a HI assay but no antibodies were detected in any of the serum samples using this method.

Western blotting was then used to test for the presence of antibodies against IBV GST-S1 in the collected serum samples. Membranes containing purified IBV GST-S1 and GST antigen (Figure 6.1A) were probed with either polyclonal rabbit anti-GST-S1 antibodies, goat polyclonal anti-GST antibodies, or pooled chicken sera collected from vaccinated chickens 28 days following vaccination. Bound antibody was then detected using the relevant secondary antibodies and chemiluminescence (Figure 6.1B). The polyclonal rabbit anti-GST-S1 antibodies and the polyclonal goat anti-GST antibodies bound to IBV GST-S1 (approximately 55 kDa) and GST only (approximately 26 kDa)
(Figure 6.1B). No antibodies against IBV GST-S1 or GST were detected in the pooled chicken sera. Similarly no antibodies against IBV GST-S1 were detected when individual chicken serum samples were tested (results not shown). The pooled chicken sera reacted with an unidentified protein slightly larger than IBV GST-S1 (Figure 6.1B). This unidentified protein was not evident in the Coomassie-stained SDS-PAGE gels that were used to assess the purity of the protein preparations (Figure 6.1A).

![Figure 6.1 Detection of anti-S1 IBV antibody. (A) Purified IBV GST-S1 (lane 1) and GST (lane 2) following SDS-PAGE and staining with Coomassie brilliant blue. (B) Western blot of IBV GST-S1 and GST probed with rabbit anti-GST-S1 antibodies (lane 1), goat anti-GST antibodies (lane 2) or pooled chicken sera collected from rILTV-IBV vaccinated birds (lane 3)](image)

6.2.2 Efficacy of rILTV-IBV

Clinical signs

No clinical signs associated with IBV infection were observed in any of challenged or mock-challenged birds over the five days following challenge.
Percentage weight gain

The percentage weight gain of female and male birds in the three different groups 3 and 5 days after challenge with IBV Vic-S are summarised in Table 6.4. The birds in group 3 showed a mean weight gain of 20.45% at day 5 post challenge which was significantly ($p = 0.015$) higher than that of the birds in group 2 (14.87%) at the same time point. No other significant differences between groups were observed.

Tracheal pathology

The tracheal gross pathology scores 3 and 5 days after challenge are summarised in Table 6.5. No significant differences between groups were observed 3 days after challenge. Five days after challenge, the gross tracheal pathology scores in the two IBV challenged groups (groups 2 and 3) were significantly ($p = 0.006$ and 0.015, respectively) higher than the scores in the mock-challenged group (group 1).

Tracheal mucosal thickness results are summarised in Table 6.6. Three days after challenge the tracheal mucosal thicknesses in the birds challenged with IBV Vic-S (groups 2 and 3) were significantly ($p = 0.001$ and 0.004, respectively) greater than that of the birds that were mock-challenged (group 1). Similarly five days after challenge there was significant differences between the tracheal mucosal thickness in the mock-challenged birds (group 1) and the IBV-challenge birds (groups 2 and 3) ($p = 0.007$ and 0.005, respectively). Tracheal mucosal thicknesses were not different between IBV-challenged birds in groups 2 and 3.
Table 6.4 Percentage weight gain 3 and 5 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>Weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Mean ± STDEV</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>3 days post challenge</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>n   Female</td>
</tr>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>2   4.36 ± 0.41</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>2   8.35 ± 0.34</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>5   8.41 ± 2.41</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Values with the same superscript letter in the same column were not significantly different (Student’s t-test)
Table 6.5 Tracheal gross pathology results 3 and 5 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>3 days post challenge Median (range)</th>
<th>n</th>
<th>5 days post challenge Median (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>10</td>
<td>0 (0-1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>0 (0-2)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>0.5 (0-1)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>10</td>
<td>2 (0-2)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>0 (0-2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9</td>
<td>1 (0-2)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Values with the same superscript letter in the same column were not significantly different (Mann-Whitney test)

Table 6.6 Tracheal mucosal thickness 3 and 5 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>3 days post challenge Mean ± STDEV</th>
<th>5 days post challenge Mean ± STDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>10</td>
<td>6.3 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.4 ± 3.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>9</td>
<td>13.0 ± 5.7&lt;sup&gt;b&lt;/sup&gt;</td>
<td>16.0 ± 4.2&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>11.0 ± 4.6&lt;sup&gt;b&lt;/sup&gt;</td>
<td>13.0 ± 3.0&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Values with the same superscript letter in the same column were not significantly different (Student’s t-test)
Tracheal histopathology results for the five parameters used to assess IBV-induced lesions present in H&E stained sections of proximal trachea are summarised in Tables 6.7 and 6.8. Three days after challenge, the score for epithelial hyperplasia/metaplasia was significantly (p = 0.034) higher in vaccinated and challenged group (group 3) than in the mock-vaccinated and mock-challenged group (group 1) (Table 6.7). The scores for inflammation were significantly higher in birds challenged with IBV Vic-S (groups 2 and 3) compared with mock-challenged birds (group 1) (p = 0.002 and 0.006, respectively) (Table 6.7). No other significant differences were observed between groups in any other parameters examined at this time point.

Five days after challenge, the scores for epithelial hyperplasia/metaplasia were significantly higher in birds challenged with IBV Vic-S (groups 2 and 3) than in mock-challenged birds (group 1) (p = 0.001 and 0.032, respectively) (Table 6.8). Similarly, the scores for inflammation in birds challenged with IBV Vic-S (groups 2 and 3) were significantly (p = 0.003 and 0.004, respectively) higher than in mock-challenged birds (group 1) (Table 6.8). The score for loss of mucus glands was significantly (p = 0.014) higher in mock-vaccinated and challenged birds (group 2) than in mock-vaccinated and mock-challenged birds (group 1) (Table 6.8). No other significant differences were observed between groups in any other parameters examined at this time point.

Replication of challenge virus in trachea

Three birds in each of the groups that were challenged with IBV Vic-S (groups 2 and 3) had detectable levels of IBV Vic-S in the tracheal mucosa, as assessed by RT-qPCR. This viral RNA was detected in the tracheal mucosal scrapings collected 3 and 5 days after challenge.
Table 6.7 Tracheal histopathology results 3 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Sloughing of epithelium</th>
<th>Epithelial hyperplasia/metaplasia</th>
<th>Inflammation</th>
<th>Loss of mucus glands</th>
<th>Loss of cilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>10</td>
<td>1 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-0)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.5 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>9</td>
<td>0 (0-2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-1)&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>1 (0-3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>0 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-1)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (0-3)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.5 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.5 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a,b</sup> Values with the same superscript letter in the same column were not significantly different (Mann-Whitney test)
### Table 6.8 Tracheal histopathology results 5 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Sloughing of epithelium</th>
<th>Epithelial hyperplasia/metaplasia</th>
<th>Inflammation</th>
<th>Loss of mucus glands</th>
<th>Loss of cilia</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>9</td>
<td>0 (0-3)(^a)</td>
<td>0 (0-0)(^a)</td>
<td>0 (0-1)(^a)</td>
<td>0 (0-3)(^a)</td>
<td>0 (0-3)(^a)</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>0 (0-3)(^a)</td>
<td>1 (0-2)(^b)</td>
<td>2 (1-3)(^b)</td>
<td>3 (1-3)(^b)</td>
<td>3 (0-3)(^a)</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>9</td>
<td>1 (0-3)(^b)</td>
<td>0 (0-1)(^b)</td>
<td>1 (1-3)(^b)</td>
<td>3 (1-3)(^a, b)</td>
<td>3 (2-3)(^a)</td>
</tr>
</tbody>
</table>

\(^a, b\) Values with the same superscript letter in the same column were not significantly different (Mann-Whitney test)
6.3 Vaccine safety and efficacy following delivery by the intra-tracheal route

6.3.1 Safety of rILTV-IBV

Clinical signs and mortalities

Clinical signs and mortalities associated with ILTV infection were observed in birds inoculated with rILTV-IBV by the intra-tracheal route (group 3). Five days after vaccination one bird died and three birds were culled (due to severe respiratory disease typical of ILT) from group 3. Two other birds from this group died, one 7 days after vaccination and one 13 days after vaccination. The bird that died 7 days after vaccination had high levels of ILTV present in the trachea. The death of the bird that died 13 days after vaccination was found to be unrelated to ILTV infection. The mortality rate in birds vaccinated with rILTV-IBV (group 3) was significantly higher than the mortality rate in mock-vaccinated birds (groups 1 and 2) (p = 0.01, Fisher’s exact test). Mortality data is summarised in Figure 6.2.

Percentage weight gain

The percentage weight gain of the birds in the three different groups over the 16 days following vaccination are summarised in Table 6.9. Birds vaccinated with rILTV-IBV (group 3) showed significantly (p = 0.005) lower percentage weight gains compared to mock-vaccinated birds in group 2, however there was no significant difference between the weight gain of the rILTV-IBV vaccinated birds (group 3) and the mock-vaccinated birds in group 1 (Table 6.9).
Figure 6.2 Bird mortality following intra-tracheal vaccination. Mortality curves of mock-vaccinated birds (♦) and birds inoculated with rILTV-IBV (●) over the 15 days following vaccination.

Table 6.9 Percentage weight gain 16 days after intra-tracheal vaccination

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>n</th>
<th>Weight gain (%) Mean ± STDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>10</td>
<td>152.2 ± 17.10&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>10</td>
<td>173.4 ± 11.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>4</td>
<td>138.8 ± 9.09&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Values with the same superscript letter in the same column were not significantly different (Student’s t-test)
Antibody detection

Sera collected at 16 days after vaccination was tested for the presence of antibodies against ILTV and IBV using ELISA. All sera from birds vaccinated with rILTV-IBV (group 3) were found to have high levels of antibodies against ILTV (mean ± STDEV ELISA titre = 208.68 ± 94.64) using the Trop-ILT ELISA. Antibodies against ILTV were not detected in sera collected from any other birds. Antibodies against IBV were not detected in any of the serum samples.

Serum samples were then used in Western blotting to detect antibodies against IBV GST-S1 protein with no antibodies detected in any of the serum samples tested (results not shown).

Replication of rILTV-IBV

To investigate the replication of rILTV-IBV following vaccination, DNA extracted from the conjunctival and tracheal swabs collected 2, 4 and 6 days after vaccination was used as template in a qPCR targeting the UL15 gene of ILTV. At all time points ILTV DNA was detected in the trachea of all birds vaccinated with rILTV-IBV (group 3) and also in a portion of conjunctival samples (5/10, 8/10 and 6/6 at 2, 4 and 6 days after vaccination, respectively) collected from these birds (Table 6.10). At both sites the highest concentration of virus was recorded 4 days after vaccination. The highest concentration of virus was recorded in the trachea. Viral DNA was not detected in any of the mock-vaccinated birds (groups 1 and 2).

Conjunctival and/or tracheal swabs were also collected from any of the birds that died or were culled due to severe clinical signs. Following DNA extraction, these samples were tested for the presence of ILTV DNA using qPCR. Viral DNA was detected in all
birds that died or were culled, except for the single bird that died 13 days after vaccination.

Table 6.10 Detection and quantitation of rILTV-IBV after intra-tracheal vaccination

<table>
<thead>
<tr>
<th>Days post vaccination</th>
<th>rILTV-IBV replication</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Trachea</td>
<td>Conjunctiva</td>
</tr>
<tr>
<td>2</td>
<td>2.95 ± 0.87 (10/10)</td>
<td>1.15 ± 0.09 (5/10)</td>
</tr>
<tr>
<td>4</td>
<td>4.63 ± 0.92 (10/10)</td>
<td>2.39 ± 0.57 (8/10)</td>
</tr>
<tr>
<td>6</td>
<td>1.98 ± 0.29 (6/6)</td>
<td>2.03 ± 0.57 (6/6)</td>
</tr>
</tbody>
</table>

6.3.2 Efficacy of rILTV-IBV

Clinical signs

No clinical signs associated with IBV infection were observed in any of the challenged or mock-challenged birds over the five days following challenge.

Percentage weight gain

The percentage weight gain of the birds in the three different groups over the 5 days following challenge with IBV Vic-S are summarised in Table 6.11. Birds of both challenged groups (groups 2 and 3) showed mean percentage weight gains that were significantly (p = 0.001 and 0.009, respectively) higher than that of the mock-challenged birds (group 1). No significant difference in weight gains was observed after challenge with IBV between mock-vaccinated birds (group 2) and those vaccinated with rILTV-IBV (group 3).
Table 6.11 Percentage weight gain at 5 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Weight gain (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>10</td>
<td>0.99 ± 3.13a</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>9.26 ± 6.27b</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>4</td>
<td>13.95 ± 12.84b</td>
</tr>
</tbody>
</table>

a, b Values with the same superscript letter in the same column were not significantly different (Student’s t-test)

**Tracheal pathology**

The tracheal gross pathology scores 5 days after challenge are summarised in Table 6.12. The tracheal gross pathology score in the mock-challenged birds (group 1) was significantly lower than those of the IBV-challenged birds (groups 2 and 3, p = 0.026 and 0.009, respectively). Tracheal gross pathology score in the rILTV-IBV vaccinated IBV-challenged birds (group 3) was significantly (p = 0.032) higher than that of the mock-vaccinated IBV-challenged birds (group 2).

Tracheal histopathology scores 5 days after challenge are summarised in Table 6.12. Only sloughing of epithelium, epithelial hyperplasia/metaplasia and inflammation were assessed. There was no significant difference between groups in the scores for sloughing of tracheal epithelium. The scores for tracheal epithelial hyperplasia/metaplasia were significantly lower in the mock-vaccinated, mock-challenged birds (group 1) compared to the birds challenged with IBV Vic-S (groups 2 and 3, p = 0.001 and 0.004, respectively). The scores for tracheal inflammation were also significantly lower in the mock-vaccinated, mock-challenged birds (group 1)
compared to the birds challenged with IBV Vic-S (groups 2 and 3, p = 0.002 and 0.003, respectively).

Tracheal mucosal thicknesses results are summarised in Table 6.13. The tracheal mucosal thickness in the birds that were mock-challenged (group 1) was significantly lower than those of the birds that were challenged with IBV Vic-S (groups 2 and 3, p < 0.001 and 0.019, respectively). There was no significant difference in tracheal mucosal thicknesses between the rILTV-IBV vaccinated birds (group 3) and the mock-vaccinated birds (group 2) after challenge with IBV Vic-S.
<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Scores for tracheal pathology</th>
<th>Histopathology</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Gross pathology</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Sloughing of epithelium</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Epithelial hyperplasia/</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>metaplasia</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Inflammation</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>10</td>
<td>0 (0-2)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>10</td>
<td>1 (0-2)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1 (0-3)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>4</td>
<td>2 (1-2)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>0 (0-2)&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a, b</sup> Values with the same superscript letter in the same column were not significantly different (Mann-Whitney test)
Table 6.13 Tracheal mucosal thickness results 5 days after challenge with IBV Vic-S

<table>
<thead>
<tr>
<th>Group</th>
<th>Vaccination</th>
<th>Challenge</th>
<th>n</th>
<th>Tracheal mucosal thickness (µm) Mean ± STDEV</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Mock</td>
<td>Mock</td>
<td>10</td>
<td>7.2 ± 1.5\textsuperscript{a}</td>
</tr>
<tr>
<td>2</td>
<td>Mock</td>
<td>IBV Vic-S</td>
<td>9</td>
<td>11.6 ± 1.6\textsuperscript{b}</td>
</tr>
<tr>
<td>3</td>
<td>rILTV-IBV</td>
<td>IBV Vic-S</td>
<td>4</td>
<td>9.9 ± 2.0\textsuperscript{b}</td>
</tr>
</tbody>
</table>

\textsuperscript{a, b} Values with the same superscript letter in the same column were not significantly different (Student’s t-test)

Replication of challenge virus in trachea

Five days after challenge, IBV RNA was detected using RT-PCR in the tracheal samples from all birds that were challenged with IBV Vic-S (groups 2 and 3, 10/10 birds and 4/4 birds, respectively). IBV RNA was not detected in tracheal samples collected from any of the mock-challenged birds (group 1).
6.4 Discussion

The safety of rILTV-IBV was assessed following vaccination of 21-day-old SPF birds with $3.0 \times 10^3$ PFU of rILTV-IBV delivered via eye-drop or intra-tracheal inoculation and its efficacy was assessed after challenging the birds with IBV. A number of parameters were measured to examine the vaccine safety and efficacy of rILTV-IBV. These included clinical signs of disease, tracheal pathology, weight gain and serum antibody response. Eye-drop and intra-tracheal routes of vaccination were assessed in separate studies. An IBV challenge protocol was also developed during this study using the Vic-S vaccine strain of IBV at 50 times the recommended vaccine dose. The safety and vaccine efficacy of the parent ΔgG-ILTV strain had been assessed previously in a series of experiments (Coppo et al., 2012; Coppo et al., 2011; Devlin et al., 2008; Devlin et al., 2007; Devlin et al., 2011; Legione et al., 2012) and these were therefore not compared with rILTV-IBV in this present study.

The results from the eye-drop study indicated that the delivery of 3000 PFU of rILTV-IBV by eye-drop vaccination was safe for 21-day-old SPF chickens as vaccinated birds did not show any clinical signs associated with ILTV infection, nor any tracheal lesions attributable to ILT. The vaccinated birds did not develop antibodies to ILTV nor had ILTV DNA in their trachea or conjunctiva 4 days after inoculation. The birds vaccinated with rILTV-IBV did show significantly lower weight gains than the mock-vaccinated birds 4 days after vaccination, however this was thought to be associated with injuries in some of the birds in this group (pecking lesions inflicted by other birds). Taken together, these results suggest that the virus did not establish infection following eye-drop inoculation at the dose administered. This is in contrast to previous studies of the parent virus (ΔgG ILTV) using the same dose and route of vaccination where mild
clinical signs of disease were noted following vaccination (Devlin et al., 2008) and viral DNA and antibodies against ILTV were detected in a proportion of the vaccinated birds 21 days following vaccination (Coppo et al., 2011).

The efficacy of rILTV-IBV after eye-drop vaccination was assessed by challenging the birds with the Vic-S strain of IBV 28 days after vaccination. Protection was assessed after challenge by examining weight gain, clinical signs of disease and tracheal pathology. None of the birds developed clinical signs attributable to IBV infection after challenge. These results are similar to those of a previous study where birds were challenged with $10^3$ median ciliostatic doses ($CD_{50}$) of IBV Vic-S after rFAV-S1 IBV vaccination (Johnson et al., 2003). In the present study, tracheal lesions, including gross and microscopic lesions, and tracheal mucosal thickness were assessed after challenge with IBV. The IBV Vic-S challenge induced pathological changes in the trachea 3 and 5 days after challenge, with the most severe lesions present 5 days after challenge. Vaccination with rILTV-IBV did not reduce the severity of the lesions after challenge when compared to the mock-vaccinated birds.

The vaccinated birds did not develop antibodies to IBV, as assessed by ELISA and HI, or to IBV S1-GST, as assessed by Western blotting. Antibodies to an unidentified protein larger than target protein IBV GST-S1 were detected in sera collected from vaccinated and unvaccinated chickens. Similar results were obtained in a previous study examining the immunogenicity of \textit{M. gallisepticum} transformants expressing the same IBV S1 gene fragment (Shil, 2010). This unidentified protein is likely to be an \textit{E. coli} protein co-purified in very small amounts along with IBV GST-S1. The SPF birds used in this study and in the previous \textit{M. gallisepticum} are free from all important primary poultry pathogens but harbour opportunistic \textit{E. coli} especially in their alimentary
system, as part of their normal intestinal flora. Thus the presence of serum antibodies to *E. coli* proteins is not unexpected.

In the second study, birds were vaccinated with rILTV-IBV via the intra-tracheal route. As replication of rILTV-IBV could not be confirmed following eye-drop vaccination in the first study, swabs were collected 2, 4 and 6 days after vaccination via the intra-tracheal route from the trachea and conjunctiva in order to better examine vaccine infection and replication. The results from qPCR analysis of these samples confirmed that rILTV-IBV replicated to high titres in the vaccinated birds. The delivery of 3000 PFU of rILTV-IBV was found not to be safe for 21-day-old SPF birds. Half of the chickens inoculated with this dose of virus died or were culled after vaccination due to severe clinical signs of ILT. This contrasts with the results from studies investigating the safety of the parent virus (Devlin et al., 2008) in which intra-tracheal delivery resulted only in mild dyspnoea in one bird. Despite rILTV-IBV replicating well after intra-tracheal inoculation, vaccination did not result in detectable antibody response to IBV 16 days after vaccination, and the birds were not protected from IBV-associated pathology 5 days after challenge. Serum antibodies against ILTV were produced, consistent with viral replication after infection. In this second experiment the weight gain results in some of the group were unexpected, particularly the low weight gains in group 1 birds after mock-challenge. The reason for this is not clear.

The reasons behind the *in vivo* phenotype differences between the rILTV-IBV (this study) and the parent strain (Coppo et al., 2011; Devlin et al., 2008) is unclear. Compared to the parent strain, rILTV-IBV appeared to show an impaired ability to replicate following eye-drop vaccination, and to cause a relatively more severe disease following intra-tracheal vaccination. It is difficult, however, to make direct comparisons
between the two viruses as the parent virus was not included in this current study. The results from the previous chapter showed that the replication and growth characteristics of rILTV-IBV and ΔG ILTV were comparable in vitro and in ovo. It appears that differences only appeared when the virus was studied in vivo. Although nucleotide sequencing revealed that there was no disruption to the sequence of the genes adjacent to the recombination region, the rest of the rILTV-IBV genome was not examined. Therefore it may be possible that mutation(s) elsewhere in the genome could have contributed to differences in the in vivo phenotype. Whole genome sequencing and/or analysis of the transcription patterns of the genes adjacent to the recombination region, (Devlin, 2006) and the other ILTV genes (Mahmoudian et al., 2012) could be undertaken to investigate potential mechanisms behind the different in vivo phenotypes of the mutant and parent viruses. It is also possible that the genetic background of the SPF flock used in this study may have influenced the in vivo behaviour of rILTV-IBV, as the SPF flock that was used to source birds for the characterisation of the parent ΔG ILTV was different to the flock used to source birds of this current study. Host genetic factors are known to influence the susceptibility of chickens to disease due to ILTV and other pathogens (Bumstead, 1998; Poulsen et al., 1998; Smith et al., 2011). Alternatively the expression of the IBV S1-N gene fragment itself could contribute to the altered in vivo phenotype of the virus.

The absence of a detectable antibody-response to the IBV protein following vaccination with rILTV-IBV, despite the vaccine virus replicating to high titres in birds vaccinated via intra-tracheal inoculation, indicates that expression of the IBV S1-N gene fragment is insufficient in its current form. The results from the in-vitro characterisation of rILTV-IBV showed that IBV S1 protein was not detected in rILTV-IBV infected cells,
although transcription of the IBV S1-N gene fragment was confirmed by RT-qPCR analysis of mRNA levels (chapter 5). This suggests that IBV S1-N protein may have been present only at very low levels in vitro. It may also be that the levels of IBV S1-N protein in vivo were too low to generate a serum antibody response, and/or that the IBV S1-N protein was not sufficiently immunogenic to generate a serum antibody response to IBV S1-N in vivo. In a previous study examining the expression of a similar IBV S1 gene fragment in M. gallisepticum, vaccination with the recombinant M. gallisepticum was able to produce IBV-S1 specific seroconversion in some of the vaccinated birds but in that study the IBV S1 protein was co-expressed with chicken inter-leukin 6 in order to enhance the host immune response (Shil, 2010). In another study examining expression of HPAI proteins in an ILTV vector, the use of a CMV promoter and synthetic introns were used to enhance protein expression and this perhaps contributed to an improved immune response induced by the expressed foreign protein (Pavlova et al., 2009). Future studies towards an improved recombinant ILTV-IBV vaccine candidate should include similar approaches described above. Particular attention should be paid to the vaccine dose and route of vaccination in light of the differences in vaccine safety observed between the two routes of administration in this study.

In addition to humoral immunity, cellular immunity has also been shown to play an important role in protecting birds from IBV infection (Collisson et al., 2000; Timms et al., 1980). Also local cell-mediated immunity in the respiratory tract, the primary target site of IBV infection, is of fundamental importance to IBV infection, as evidenced by the protection induced by transfer of IBV infection-induced T cells from challenged birds (Seo et al., 2000). In the present study, the role of cell mediated or local immune
responses in protection after vaccination were not examined but will be of interest in future studies.

The challenge protocol in the current study used the IBV vaccine strain Vic-S, which was also the source of IBV S1-N gene in rILTV-IBV. For challenge the Vic-S strain was used at 50 times the vaccine dose recommended by the manufacturer. In both studies, the challenge protocol induced pathological changes in the trachea of challenged birds but did not cause clinical disease. Measuring tracheal mucosal thickness has been utilised in a previous study on IBV (Chousalkar et al., 2007) but a detailed scoring system to assess different histopathological parameters in the trachea, which was developed and implemented in our study, has not been reported before. The parameters of tracheal epithelium hyperplasia/metaplasia, inflammation and loss of mucous glands were found to be the most useful parameters for assessing IBV-induced microscopic lesions, and are likely to be valuable for future IBV experiments.

Persistence of IBV in the trachea after challenge was assessed by RT-qPCR or conventional RT-PCR, with conventional RT-PCR detecting IBV RNA in a higher proportion of challenged birds compared to RT-qPCR. This may reflect a difference in sensitivities between the two assays, although the birds in the two studies were challenged at slightly different ages which may have produced this result. It is important to note that the detection of viral RNA using these methods does not measure the amount of infectious challenge virus in the trachea (Johnson et al., 2003) thus in circumstances where it is important to quantitate infectious IBV alternative methods such as virus isolation and titration by EID$_{50}$ assay should be utilised.
In future studies utilising the ΔgG ILTV strain as a vaccine vector it may be helpful to use the complete IBV S1 gene as the inclusion of the three main neutralising epitopes in S1 may contribute towards better protection against IBV, as observed in other studies (Johnson et al., 2003; Shil et al., 2011; Song et al., 1998b). This could potentially be performed in conjunction with the use of exogenous promoters, synthetic introns and co-expression of immune-modulating molecules, as outlined above.
7 General discussion

The first aim of these studies focussed on developing novel DIVA diagnostic tools to differentiate birds vaccinated with recombinant ΔgG ILTV from birds infected with WT ILTV. The second aim of these studies focussed on using the ΔgG ILTV as a vaccine vector to express regions of the S1 and N genes of IBV (rILTV-IBV). Both these aims have the potential to add value to the ΔgG ILTV vaccine candidate and ultimately have the potential to improve disease control in poultry flocks.

The ELISA and TaqMan qPCR developed in these studies were both designed to act as companion diagnostic tools with the ΔgG ILTV candidate vaccine. The recombinant gG based ELISA was able to detect and differentiate commercial birds exposed to ILTV (by vaccination with a conventional ILTV vaccine) from unexposed birds. Under these conditions the performance of the gG based ELISA was comparable to that of a commercial ILT ELISA. The gG based ELISA did not perform as well when applied to serum samples from SPF birds infected with different strains of ILTV under experimental conditions, including birds infected with the ΔgG ILTV. This is currently the only source of serum for birds infected with ΔgG ILTV as this vaccine candidate is not yet approved for use outside the laboratory. It will be important to revaluate the performance of this ELISA using serum samples from birds infected in the field with ΔgG ILTV once such samples become available. ILTV ELISAs are well suited for monitoring the infection status at the level of the flock (Meulemans and Halen, 1982; Sander and Thayer, 1997), which is consistent with the intended use of the gG-based ELISA in DIVA disease control programs. The gG-based ELISA will need to be paired with an ELISA utilising another ILTV antigen in order to correctly serologically determine the infection status of a flock (i.e. infected with ΔgG ILTV or another strain.
of ILTV). This could utilise whole virus, as is used in some currently available commercial ELISAs, such as the Trop-ILT ELISA, or potentially another ILTV antigen, such as glycoprotein E or gp60 (Chang et al., 2002).

A TaqMan PCR to detect and differentiate ΔgG ILTV from WT ILTV was also developed in this study. Recently, real-time PCR assays have been shown to be rapid, sensitive and able to quantify viral nucleic acid directly from clinical samples (Mackay et al., 2002). The real-time TaqMan PCR developed in this study was able to detect and quantify DNA template from samples collected from SPF chickens following infection with ΔgG ILTV or WT ILTV under experimental conditions. The assay was also able to detect DNA from a number of different vaccine and field strains of ILTV and therefore could potentially be beneficially used as a general diagnostic test for ILTV infection. The detection limit of the assay of 10 viral template copies for ΔgG and WT ILTV compares favourably with other ILTV qPCRs (Callison et al., 2007a; Mahmoudian et al., 2011).

The TaqMan qPCR assay was tested in individual and multiplex formats and was found to perform well in both these formats. The multiplex assay was able to detect both ΔgG ILTV and WT ILTV in cultured LMH cells and embryonated hen eggs that were co-infected with both viruses. This suggests the assay could be useful for detecting and quantititating mixed infections in both field and research settings. In particular the multiplex assay could be used to assess how well the ΔgG ILTV vaccine protects against replication of virulent wild type virus after challenge. Traditionally it has been difficult to discriminate between replication of ILTV challenge virus from replication of ILTV vaccine virus due to the genetic similarities between virus strains (Bauer et al., 1999).
Whilst the ELISA is best suited for use in later stages of an outbreak of disease (after the appearance of serum antibody) (Crespo et al., 2007; Sander and Thayer, 1997), the Taqman qPCR may be best suited for use in the earlier stages of a disease, when virus is shed in larger numbers (Guy et al., 1992; Hughes and Jones, 1988). A comprehensive evaluation of how to best use the TaqMan PCR and the gG-based ELISA tests in combination is necessary and could be conducted after the candidate ΔgG ILTV vaccine has been permitted to use in field. This would allow the efficacy of these DIVA diagnostics to be better evaluated and would provide a better understanding of how these tools may be applied to help control disease. Together the use of these DIVA diagnostic tools, alongside the use of the ΔgG ILTV vaccine, has the capacity to significantly contribute towards future ILT control and eradication programs, beyond what can be achieved with currently available ILT vaccines (Coppo et al., 2013).

The recombinant virus (rILTV-IBV) that was developed and tested in this study demonstrated that there is the potential to use the ΔgG ILTV strain as a vaccine vector. The insertion of the IBV S1 and N gene fragment into the ΔgG ILTV genome resulted in expression of the IBV S1 and N gene fragment (as confirmed by increasing levels of IBV S1-N gene transcripts over time in infected LMH cells) but did not alter the growth characteristics of the virus in vitro or in ovo. The ability to grow ILTV vaccines to high titres in vitro or in ovo is important if the vaccine is to be produced in sufficient quantities for large-scale use (Guy et al., 1991). Preliminary testing in chickens showed that some aspects of the in vivo phenotype of rILTV-IBV appeared to differ from that of the parent virus, as assessed in previous studies (Coppo et al., 2011; Devlin et al., 2008). In particular the virus did not appear to replicate following eye-drop inoculation at the dose studied, but did replicate following intra-tracheal vaccination, however this route
of administration was associated with increased signs of disease. The reasons for these differences were not investigated in this study. Future investigations into the influence of host genetic factors, and studies to elucidate if there are any alterations in other regions of the virus genome may shed light on this observation.

In addition to the investigation into the pathogenicity of rILTV-IBV, these studies also investigated the immunogenicity of rILTV-IBV. Intra-tracheal inoculation of rILTV-IBV did not protect birds from infection nor tracheal pathology following subsequent challenge with IBV Vic-S. Furthermore, prior to challenge the vaccinated birds did not produce detectable levels of antibodies to IBV S1. It was speculated that the undetectable levels of foreign protein expression observed in the initial characterisation of rILTV-IBV in vitro may also be responsible for the lack of induction in protective immune response in-vivo. Further investigations are warranted to examine this. An improved recombinant ILTV-IBV construct with enhanced S1 protein expression may help in inducing an enhanced immune response to vaccination, which may correlate with protection from disease following challenge. There are a number of ways that this could be achieved but inclusion of a stronger promoter, such as a CMV promoter (Pavlova et al., 2009), or even another endogenous promoter already present within the ILTV genome, appears logical for this future work. Inclusion of the entire S1 and N genes, rather than just a portion of the S1 gene, may also help to induce a stronger immune response.

Vectored vaccines have the potential to improve control of disease in poultry flocks. Their advantages over conventional vaccines include the potential to reduce the number of vaccines that are delivered to poultry flocks, and the potential to reduce the number of vaccine strains present in commercial flocks. This would be important for vaccine
strains that can contribute to clinical disease through reversion to higher levels of virulence or recombination (Lee et al., 2012; Moormann et al., 2000; Song et al., 1998b). Vectored vaccines also have the potential to be used in conjunction with DIVA diagnostic tools (de Boer et al., 1990; Godoy et al., 2013; Iqbal, 2012; Pavlova et al., 2010; Starick et al., 2006). In future studies it may be possible to combine an improved recombinant ILTV-IBV vaccine with DIVA diagnostic tools, using similar approaches to those employed in the development of the DIVA TaqMan PCR and ELISA in this study. The narrow host range of ILTV (Guy and Garcia, 2008) is advantageous for controlling the spread of any genetically modified viruses utilising an ILTV vector into other species. To date the use of ILTV as a vector has been investigated only in a relatively small number studies (Luschow et al., 2001; Pavlova et al., 2009; Veits et al., 2003) but continued research and development in this area represents a worthwhile area of investigation to enhance disease control in poultry in the future.
8 References


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Zhang, H.M., Hunt, H.D., Kulkarni, G.B., Palmquist, D.E., Bacon, L.D., 2006. Lymphoid organ size varies among inbred lines 6(3) and 7(2) and their thirteen recombinant congenic strains of chickens with the same major histocompatibility complex. Poult Sci 85, 844-853.


Appendices

Appendix 1. Optimised gene sequence of ILTV gG

GGATCCGGTTCTGCCCCGGTCCTGGATGGCCTGGAAAGCAGCCCGTTCCCGTTTGGTGGA
AAATCATCGCGCAAGCGTGTAACCGTACTACCATTGAGGTCACGGTGCCGTGGAGCGAC
TACAGCGGTTGCGGCTGATTTCACAACCTGAACGCGAGCGCGTGCTGCAAGCGATGCT
GGTATCCCTGTTGGGCTGATTTCGCAAGCAGAAAGGTTCCAGTGCTGTTTGGTGACG
GTAGCTGGGTAGTACGTTAGCGGCAATGGTTCTGGTACTGGTGTAATTCTAGGTACG
ATGCAGGTATTTTACGCTATCACGTGTCGGTGGTAAAGGCTACACCGGCTCTGCTG
TACTTGAATTTGTCGCAACCCGGTGCACCGACCATTAGCTACTACGTTAGCGGACG
GTCCGATTGGAGAAGATTTAGCAATTGCTGGTATGCGCCGTTGCCCGTGGACC
ACCGTTGCAAGCAGCTGTTGAAAGAACAAGAGAACCGGACGCTGTACGCTCTGTG
GGACGAGGATGTGACGGCAGGCTGAGCTACGTTGGATAGAAGCTCAGGAAACCAGTC
CGCGCATGGTTGCTTCAAGCCGCAAGCAACCTTGCAACAGAGCCGCTACACCACCAT
CACCACGTATCTAGAgtcgac

The restriction endonuclease enzyme sites are highlighted with lower case letters

*BamHI* (ggatcc) and *SalI* (gtcgac)
Appendix 2. Analysis of protein expression in LMH cells infected with rILTV-IBV or ΔgG ILTV using IFA. LMH cells were fixed 1 day after infection and incubated with either monoclonal antibody to ILTV or rabbit anti-GST-IBV S1 polyclonal serum. Bound antibodies were detected by FITC-conjugated antibodies and visualised on a fluorescent microscope.
Author/s:
Shil, Niraj Kanti

Title:
Development of a novel recombinant vaccine and diagnostic tools for control of infectious laryngotracheitis

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2014

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