Studies on immunopathogenesis and diagnosis of infectious bronchitis virus in chicken

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Abstract

An RT-PCR assay was developed to amplify and sequence the full S1 gene of classical and variant infectious bronchitis virus (IBVs) enriched in allantoic fluid (AF) or the same AF inoculated onto Flinders Technology Association (FTA) cards. Seven IBV strains (M41, D274, 793B, IS/885/00, IS/1494/06, Q1 and QX) were grown in SPF eggs and RNA was extracted from AF. Full S1 gene amplification was achieved by using two primers and products were sequenced using primers; A, SX3, 1050+ and 1380+ to achieve full S1 gene coverage. Following serial dilutions of AF, it was found that detection limits of the partial assay were higher than those of the full S1 gene. Partial S1 sequences exhibited higher than average nucleotide similarity percentages (79%; 352bp) compared to full S1 sequences (77%; 1,756bp), suggesting that the full S1 protocol has greater strain differentiation efficiency. For IBV detection from AF inoculated FTA cards, four serotypes were incubated and stored for up to 21 days at three temperatures; 4°C, room temperature (24°C) and 40°C. RNA extracted and tested with partial and full S1 protocols. Through partial sequencing, all IBVs were successfully detected at all sampling points and storage temperatures. In contrast, for full S1 sequencing, was not able to amplify the gene beyond 14 days of storage or when stored at 40°C. Full S1 sequencing appears to be suited for detection of IBVs enriched in AF, and has limited application for samples directly embedded onto FTA cards.

Following Q1-like infection of specific pathogen free (SPF) or two different breeds of broiler chicks, the live body weight of all three types of chicks were significantly reduced. Also, respiratory clinical signs were found in all types of chick. For the infected SPF chicks, all swabs were RT-PCR positive, with the IBV viral load peaking in the trachea and kidneys at 9 dpi, whereas the proventriculus peaked later at 14 dpi. Significant up-regulation in the expression of several genes (IFNα, TLR3, MDA5, LITAF, IL-1β and IL-6) were seen in the trachea and kidneys at 3, 7 and 9 dpi. In the broiler chicks, the immunopathogenesis of Q1 was cross-compared between fast and slow growing broiler birds. For the fast-growing line (Line-A), swabs from the infected group were RT-PCR positive at all sampling days, whereas the slow growing line (Line-B) were positive until 14 dpi. At 7-9 dpi, higher viral loads were found in the trachea, proventriculus and kidney of fast growers compared to slow growers. Mean IBV ELISA antibody titres in the Line-B were higher than Line-A. Tracheal innate immune response showed IFN-α up-regulation only in Line-A but IFN-β was up regulated in both lines. For TLR3, an up-regulation was seen in Line-A up to 7 dpi, and for all sampling days in Line-B. MDA5 was up-regulated in Line-A and down-regulated in Line-B at 1 dpi. In the kidneys, for Line-A birds, IFN-α and IFN-β were up-regulated at 1 and 1-3 dpi respectively. There was up-regulation in TLR3 in Line-B throughout the study period but not for Line-A. MDA5 was up-regulated in both lines at 7 and 9 dpi. It appears that the immunopathogenesis of IBV Q1 infection in slow growing (Line-B) chicks was milder in terms of the proinflammatory cytokines levels when compared to the fast growers (Line-A), which could be associated with the genetic differences between these breeds.

In an attempt to establish an *in vitro* infectious model to cross-compare the virulence of IBV live vaccines, eight vaccines and three virulent strains of IBVs were assessed in tracheal organ cultures (TOCs). At 24 and 72 hours post infection (hpi), TOC media and tracheal rings were collected and used for RT-PCR, qRT-PCR, measurement of innate immune responses and examination of total apoptotic cells. Differences in virulence were noted between the strains as certain vaccine strains resulted in cilia destruction comparable with the virulent strain. Average cilia motility readings showed that Mass1 and virMass reached complete ciliostasis at 72 and 96 hpi respectively, whereas, in the 793B and QX groups complete ciliostasis was reached for all strains by 120 hpi. The qRT-PCR analysis revealed decreased viral presence in the media at 24 and 72 hpi. Differences were found between the total apoptotic cell counts in the tracheal rings among virulent and vaccine strains. Downregulation in mRNA expression of IFN-α at 24 and 72 hpi occurred in all virulent and vaccine infected TOCs. At 24 hpi, there was up-regulation in IFN-β which was down regulated by 72 hpi in virulent infected TOCs. At 24 and 72 hpi, there was up-regulation in the mRNA expression of TLR3 in all vaccine and variant strains. An up-regulation of MDA5 was seen at 24 hpi in Mass serotype strains, vir793B, 793B1 and QX strains. This study demonstrates successful use of the *in vitro* TOC model for distinguishing differences in virulence and replication rates among the vaccine IBV strains.

Four of the vaccine strains used above were included in an *in vivo* experiment to validate previous data using a chicken host model. Following inoculation of different vaccine strains in SPF chicks, the immunopathogenesis was evaluated. IBV vaccines (Mass1, Mass2, 793B1 and 793B2) were administered by the oculo-nasal route and chicks were monitored daily for clinical signs. At 1, 3, 5, 7, 9 and 14 dpi, OP and CL swabs were collected for virus detection, and blood was collected for antibody assay. Necropsy was carried out on 1, 3, 5, 7, 9 and 14 dpi, with gross lesions observed and tracheal tissues collected for qRT-PCR, immunohistochemistry (CD4+ and CD8+), histopathology and host innate immune gene expressions. Respiratory signs started from 3 dpi. Viral load was lower in the 793B1 group at 5-7 dpi, compared to other groups. Higher expression of IFN-β was seen at 3 dpi in 793B groups, whereas793B2 showed a lower expression of TLR3 at 5-7 dpi compared to other groups. Down-regulation of IL-6 was seen at 7-9 dpi in all inoculated groups except for Mass1. There was higher up-regulation of IFN-γ at 7-9 dpi in Mass1 and Mass2 compared to the control and 793B1 and 793B2. From lachrymal fluid, tracheal washes and tracheal tissue there was higher up regulation in IgG expression in the all inoculated groups at 9-14 dpi. In Mass1 and 793B1, there was higher up-regulation of IgA in the tracheal tissue at 7-9 dpi. The 793B2 inoculated groups demonstrated higher cell mediated immune responses, represented by higher CD8β gene expression in the period of 5-9 dpi, and higher cell counts of CD4+ and CD8+ at 14 dpi. Results demonstrated that the *in vivo* were generally comparable to *in vitro* findings. This demonstrates that large number of IBV live vaccines could be screened for virulence and early immune responses using TOCs, instead of birds.

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Declaration

The data presented in this thesis has been obtained in either *in vivo* or *in vitro* experiments carried out by myself in the University of Liverpool (Leahurst Campus) as a member of avian respiratory disease group. I played a major role in the preparation and performance of the experiments, and subsequent data analysis and interpretations were entirely undertaken by myself. Any contributions from technicians' or colleagues in the collaboration are stated. This work has not been submitted for any other degree or professional qualification in any other University or other institute of learning.

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Basim Manswr

September 2018

Dedication

This work here is dedicated to my mother, she always says “Just keep going, you are different from others” without her, I would not be here. Also, dedicated to my brother as well as my entire family for their kindness and support.

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Carrying out an *in vivo* and *in vitro* poultry trial and investigation is always fraught with difficulty, and requires huge energy and commitment: this current thesis was no exception. It would have been impossible to complete this work alone and I am grateful for the advice and help I have received from other people over almost 4 years of study. First of all I would like to express my thanks and appreciation to Dr Kannan Ganapathy for persevering with me as my primary supervisor throughout the time it took me to complete this research and write my thesis. My inspiration for doing this research came from his plans, thoughts, advice, direction, feedback and support. I am grateful for his kindness and encouragement at both the personal and academic levels. I am also grateful to Professor Julian Chantrey, who has generously given his time and expertise to improve my work; I will never forget his help with histopathology and immunohistochemistry. I appreciate Dr Christopher Ball for his contribution and his good-natured support, especially for his help with analysing DNA sequences, useful feedback, and writing efforts over the years. I need to express my gratitude and deep appreciation to Mrs Anne Forrester whose friendship, hospitality, knowledge, and wisdom have supported me throughout the entire study. Thankyou also to Mrs Sue Jopson for her help during the chicken experiments. I must acknowledge many of my friends and colleagues, other students and technicians who assisted, advised, and supported my research and writing efforts over the years.

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List of publications and presentations

Below are the published papers peer-reviewed scientific journals and presentation and conferences attended as either poster or presentation come from work which have carried out in this thesis.

Publications in peer-reviewed scientific journals

- Manswr, B., Ball, C., Forrester, A., Chantrey, J. and Ganapathy, K. (2018) 'Evaluation of full S1 gene sequencing of classical and variant infectious bronchitis viruses extracted from allantoic fluid and FTA cards', Avian Pathology, 1-21.
- Manswr, B., Ball, C., Forrester, A., Chantrey, J. and Ganapathy, K. (2018) 'Infectious bronchitis virus Q1 causes greater tracheal than renal pathology and innate immune responses in specific pathogen free chicks', submitted manuscript, Virology.
- Manswr, B., Ball, C., Forrester, A., Chantrey, J. and Ganapathy, K. (2018) 'The immunopathogenesis of infectious bronchitis virus Q1 in slow and fast growing commercial broiler chicks', in -preparation manuscript, Microbial Pathogenesis.

Conference Publication

- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Immunopathogenesis of infectious bronchitis virus Q1 in SPF chicks. 9th International Symposium on Avian Corona- and Pneumoviruses, Utrecht, The Netherlands, 21-24 June 2016. Pp128-133.
- C. Ball, B. Manswr, A. Forrester, J. Chantrey and K. Ganapathy. Full versus partial S1 gene sequencing of classical and variant avian infectious bronchitis viruses. 9th International Symposium on Avian Corona- and Pneumoviruses, Utrecht, The Netherlands, 21-24 June 2016. Pp156-161.

Presentations and posters at national/ international seminar/meetings

- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Immunopathogenesis of infectious bronchitis virus Q1 in SPF chicks. 9th International Symposium on Avian Corona- and Pneumoviruses, Utrecht, The Netherlands, 21-24 June 2016. (Oral presentation).
- C. Ball, B. Manswr, A. Forrester, J. Chantrey and K. Ganapathy. Full versus partial S1 gene sequencing of classical and variant avian infectious bronchitis viruses. 9th International Symposium on Avian Corona- and Pneumoviruses, Utrecht, The Netherlands, 21-24 June 2016. (Oral presentation).
- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Variations in the immunopathogenesis of infectious bronchitis virus Q1 in commercial broiler chicks with different growth rates. World Veterinary Poultry Association Conference at Edinburgh, 4-7 September 2017. P. 364. Poster PO-VRD-14.
- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Variations in immunopathogenesis of infectious bronchitis virus Q1 in commercial broiler chicks with different growth rates. Liverpool, University, Infection and Global Health Day.16 November 2017. Poster 28.
- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Pathobiology of newly emerged infectious bronchitis virus strain Q1 in specific pathogen free chicks Liverpool, University, Infection and Global Health Day.17 November 2016. Poster No.15.
- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Full S1 gene sequencing of classical and variant avian infectious bronchitis viruses. Liverpool, University, Infection and Global Health Day.25 November 2015. Poster No.11.

- B. Manswr, C. Ball, A. Forrester, J. Chantrey and K. Ganapathy. Pathobiology of newly emerged infectious bronchitis virus strain Q1 in specific pathogen free chicks Liverpool University, Faculty Poster Day.19 June 2017. Poster No.17.
- M. Al-Rasheed. B. Manswr, C. Ball, A. Forrester, G. Leeming, K. Ganapathy, Immune kinetics in the turbinate and trachea of vaccinated and unvaccinated broiler chicks following IBV M41 challenge. GARAD conference, Hanoi, Vitenam.17-19 January (Oral presentation).

List of abbreviations

18S	18S ribosomal RNA
Ab	Antibody
ABC	Avidin biotin complex
AF	The agar gel-precipitating test
H Ag	Heterologous antigen
AGP	Agar gel precipitation
AIBV	Avian Infectious bronchitis virus
APC	Antigen-presenting cells
Ark	Arkansas
ARMS RT-PCR	Amplification refractory mutation system RT-PCR reaction
b ELISA	Blocking enzyme linked immune sorbent assays
BLAST	Basic Local Alignment Search Tool
bp	Base pairs
BSA	Bovine serum albumin
CALT	Conjunctiva-associated lymphoid tissue
CAMs	Chorioallantoic membranes
CD50	Ciliostasis dose 50
CD8+ T	Cluster of differentiation 8
cDNA	Complementary DNA
CE	Cytopathic effect
CMI	Cell-mediated immune
Conn	Connecticut
CPE	Cytopathic effect
Ct	Threshold cycle
CTLs	Cytotoxic T cells
DAB	3, 3 –diaminobenzidine
DC	Dendritic cells

De	Delaware
dH2O	Distilled water
doa	Days of age
dpi	Day post infection
DPV	Day post vaccination
DPX	Distyrene Plasticizer Xylene (DPX
dsRNA	Double-stranded (ds) RNA viruses
DTT	Dithiothreitol
E. coli	*Escherichia coli*
EID50	50% Egg Infective Dose
EID50/ml	50% Egg Infective Dose/ml
ELISA	Enzyme linked immune sorbent assays
EM	Electron microscopy
EP	End point
EXO	Exonuclease
FCR	Food conversion rate
FITC	Fluorescein Isothiocyanate
H&E	Haematoxylin and Eosin stain
HG	Hadrian gland
HI	Hemagglutination
hpi	Hours post infection
HPR	Horseradish peroxidase
HRM	High-resolution melt curve analysis
HS	Hematoxylin stain
I ELISA	Indirect (I ELISA)
IB	Infectious bronchitis
IBD	Infectious bursal disease
IBV	Infectious bronchitis virus

IFA,	Immunofluorescence
IFN-γ	Interferon gamma
IgA	Immunoglobulin A
IgA	immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IgY,	Immunoglobulin Y(G)
IHC	Immunohistochemistry
IL1β	Interleukin 1-β
IL6	Interleukin-6
INF I	Interferon
IPA	Immunoperoxidase assay
IT02	Italy O2
Kb	Kilo base
KG3P	Kannan Ganapathy third passage
LITAF	Lipopolysaccharide-induced tumor necrosis factor (TNF)-α
LMA	Agarose low melting temperature
lpb ELISA	Liquid Phase Blocking
M EGA6	Molecular Evolutionary Genetics Analysis
MAbs	Monoclonal antibodies
Mass	Massachusetts
MDA	Maternal derived antibodies
MDA5	Melanoma differentiation associated protein 5
MDV	Marek's disease virus
MEM	Minimum Essential Medium
MFL	Median fluorescent level
MHC	Major histocompatibility complex
Mm	Millimeters

Mn 2+	Manganese cation (2+)
mRNA	Messenger RNA
Mx	Myxovirus-resistance protein
Myd88	Myeloma differentiation protein 88
NCBI	National Centre of Biotechnology Information
NDV	Newcastle disease virus
ng	Nano gram
NGS	Next generation sequencing
NK	Natural killer cells
NLRs	NOD-like receptors
nrTW	Naturally recombinant
NOD	Nucleotide-binding oligomerisation domain like receptors (NLRs)
^{o}C	Celsius degree
ODs	Optical density
OCT	Optimal cutting temperature compound
OP or CL	Oropharyngeal or cloacal swabs
OIE	World Organisation for Animal Health
PAS	Periodic acid–Schiff
PBS	Phosphate buffer saline
PCA	Peripheral ciliary activity
PFA	Paraformaldehyde
PFU	Plague forming units
Pmol	Picomoles per litre
PRRs	Pattern recognition receptors
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RCA	Relative ciliary activity
REU	Relative equivalent units
RFLP	Restriction Fragment Length Polymorphism

RIG-I (RLRs)	retinoic acid-inducible gene 1 like receptors
RLRs	RIG-I-like receptors
RNA	Ribonucleic acid
Rpm	Round per minuets
RPMI	Roswell Park Memorial Institute
RT	Reverse transcriptase
RT-LAMP	Reverse transcription loop mediated isothermal amplification
RT–PCR	Reverse transcription polymerase chain reaction
S ELISA	Sandwich (S ELISA
S/p ratio	Sample/positive ratio
SAP	Shrimp alkaline phosphatase
sIg	Secretory immunoglobulins
SNP	Single nucleotide polymorphism
SPF EGE	Specific pathogen free egg chicks embryo
Spp	Species
T cell	T lymphocyte(cell)
TBE buffer	Tris-borate-EDTA
TCoV	Turkey coronavirus
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TOCs	Tracheal organ cultures
TPS	Tris buffer saline
TRIF	TIR-domain-containing adapter-inducing interferon-β)
TUNEL	In situ Terminal deoxynucleotidyl transferase dUTP nick end labelling assay
U/µl	Unit to microliter
USP/ml	United States Pharmacopeia/ milliliter
UTR	3'untranslated region gene
VI	Viral neutralisation

VNT	Virus neutralisation test

Chapter 1: Introduction and literature review

1.1 Introduction

Infectious bronchitis (IB) is an avian viral disease; it is an important acute disease, and it is associated with respiratory disorder in domestic chickens caused by infectious bronchitis virus (IBV), a highly transmissible coronavirus belonging to the family *Coronaviridae*. IBV was first isolated in 1936, with the first commercial vaccine against the Massachusetts serotype following in 1950 to help reduce economic losses. In 1956, the first available report was published confirming that IBV has several serotypes (Raggi and Bankowski 1956). Since the initial discovery, there have been many reports concerning the disease and its prophylaxis, but it remains one of the most important poultry diseases in the world, with the virus mutating into new serotypes and strains requiring the development of new empirical vaccines (Dolz *et al.*, 2008).

Schalk and Hawn (Schalk and Hawin 1937) defined an apparently new breathing disease among chicks in the U.S. state of North Dakota in 1931. Since that time, IB has been recognised in broiler, layer and breeder chickens internationally, and more than 50 serotypes or variants have been discovered (Cavanagh and Gelb 2008; Jackwood 2012; Jackwood and de Wit 2013). The highly contagious nature of IBV and emergence of numerous serotypes have complicated IB control with vaccination (Jackwood and de Wit 2013). The arrival of novel IBV strain variants presents an ongoing problem for the poultry industry (Hofstad 1984), as these variants may circulate and persist among both vaccinated and non-vaccinated flocks (Cavanagh and Gelb 2008).

IBV is a single-strand RNA virus of approximately 27.6 kb in size (Boursnell *et al.*, 1987). The spike protein comprises 1145 amino acids, and its purposes include attachment to the host cell and neutralisation of antibodies (Abs) yet it initiates host protective immunity (Ignjatovic and Galli 1994; Johnson *et al.*, 2003). The IBV spike protein undergoes post-

translational cleavage to form S1 and S2 subunits (Cavanagh and Davis 1986). It is the most variable of the IBV proteins, with high levels of mutation and recombination events mapping to the S1 region (Adzhar *et al.*, 1997; Kingham *et al.*, 2000).

IBVs have an affinity for respiratory or gastrointestinal epithelium, and cause relatively mild clinical signs in adults but with more severe symptoms occurring in young birds (Cavanagh 2007). The virus replicates in the cytoplasm of host cells, where the RNA works directly as messenger RNA (mRNA) for the RNA polymerase (Hiscox *et al.*, 2001). The S1 subunit binds to host cell receptors, which are responsible for stimulating neutralising Abs (Cavanagh and Davis 1986); in addition, the S1 subunit is important for cell tropism (Casais *et al.*, 2003). The more variable S1 section is the most significant for this induction (Casais *et al.*, 2003; Winter *et al.*, 2008). The virus is assembled and buds into the endoplasmic reticulum; subsequently, it is released through exocytosis. After exocytosis, virions remain attached to the cellular membrane (Murphy *et al.*, 1999; Hodgson *et al.*, 2006).

The economic importance of IBV was first observed by Delaplane and Stuart in 1939, who identified IB as a common respiratory disease in semi-mature and older chickens and could result in significant losses in egg production (Delaplane and Stuart 1939). These losses are well documented which result from infection alone or further to secondary infection with another pathogen such as *Escherichia coli*. Such losses are from mortalities, medication, delayed growth, infertility and eggshell deformities. Eventually, condemnation of a large number of carcasses could be the result of IBV infection reported in the slaughterhouse (R. C. Jones 2010). Due its adverse effects on poultry production and also its negative role on economic trade, because of the potential for viral spread, the World Organisation for

Animal Health (OIE) has defined IBV as an internationally important disease of high concern (Bagust 2013).

Recent advancements in molecular identification and sequencing techniques have allowed for new strains and variants to be identified (Sjaak de Wit *et al.,* 2011; Jackwood 2012). According to international diagnostic laboratory, IBV genotyping is important for controlling infection through selection of the most appropriate vaccine.

It is important to use appropriate diagnostic methods to differentiate IBV from other upper respiratory pathogens, such as avian influenza and Newcastle disease. Although viral isolation is considered the gold standard for IBV diagnosis, it is time consuming, laborious and requires several passages in embryonated chicks (J. Gelb, Jr. and M.W. Jackwood 1998). The detection of IBV can be confirmed from infected chicken tissue by immunohistochemistry (IHC) (Chen *et al.,* 1996) or in situ hybridisation (Collisson *et al.,* 1990). Recently, reverse transcription polymerase chain reaction (RT-PCR) has proven to be a useful and rapid technique for IBV diagnosis and it has allowed for strain genotyping through sequencing (De Wit 2000). Moreover, viral dissemination can be detected and quantified in the infected tissues using real-time polymerase chain reaction (Real-Time PCR), also known as quantitative polymerase chain reaction (qPCR), during the period of infection, even if virus isolation has not been successful (Roh *et al.,* 2014).

In the 1950s, basic methods were used for IBV isolation and identification, including serum neutralisation testing in embryonated eggs (Fabricant and Levine 1951). After inoculation, usually at 9 days of embryonic life, viral isolation in the embryonated eggs typically resulted in curled and stunted embryos. These signs become more prominent after 7 days post-inoculation, illustrating the presence of IBV infection. However, this process, which used field samples, required serial passages in embryonated eggs; thus, the results

could be misinterpreted, as the findings were relatively subjective and false-positive results may have occurred. Although this technique was still being used in recent work (Cook *et al.*, 2012), this classical method is time consuming, expensive and requires a significant number of inoculated chick embryos. To increase the sensitivity of RT-PCR, specialists are now using *in vitro* systems to amplify the virus. Several additional procedures have now been adopted to identify or differentiate IBV, such as restriction fragment length polymorphism (RFLP) (Abreu *et al.*, 2006), hybridisation (Nagano *et al.*, 1993) and sequencing (Krapez *et al.*, 2011).

For molecular detection, samples of swabs and tissues, or imbedded onto Flinders technology association (FTA) cards are processed for conventional RT-PCR (Moscoso *et al.*, 2005; Cook *et al.*, 2012). However, the use of RT-PCR alone does not discriminate as to whether there is viable or non-viable virus in the chicken at the time of sampling. This feature is crucial when analysing PCR results in connection with the disease situation in the field. In addition, RT-PCR does not provide live viruses that can be used for challenge studies or to produce new vaccines (Cook *et al.*, 2012).

Some IBVs are established, others have recently emerged and still others appear for a short period and then disappear from that area, often reappearing in a different region. Despite differences in IBVs' identity, infection with these viruses causes similar signs at two sites of infection, taking respiratory and urogenital forms. Different diagnostic approaches for IBV have been reviewed in previous work (De Wit 2000; Ignjatovic and Sapats 2000; Ganapathy 2009; Villarreal 2010; Bande *et al.*, 2016). This section presents a review of recent literature on some aspects of IBV, including the available diagnostic assays, which focus on clinical and laboratory diagnosis, pathogenesis and immunity.

1.2 The Virus

The aetiological agent of IB is a Gammacoronavirus, genus *Coronaviridae*, order *Nidovirales*. The viral particle is constituted by an outer envelope with glycoprotein projections, the helicoidal symmetry nucleocapsid contains the 27,6 kb single strand RNA; the genomic material of the virus is made up of open reading frames (ORFs) and genes. Gene 1, which accounts for approximately two-thirds of the genome, encodes many proteins that are associated with RNA replication and transcription. The structural protein genes encode for spike (S), small envelope (E), matrix (M) and nucleocapsid (N) proteins. Genes that encode non-structural (ns) proteins are situated among the structural protein genes and their numbers differ among Coronaviruses (Cavanagh 2005), with IBV having two such genes (3 and 5) (Cavanagh 2007). Gene 5 is functionally bicistronic and encodes two open reading frames (ORFs), 5a and 5b, which are expressed in IBV-infected cells. Gene 3 is functionally tricistronic, having three ORFs (3a, 3b, and 3c) and it is the third ORF, 3c, which encodes the E protein of IBV (Cavanagh 2007).

1.3 IBV variants distributions

It is well known that IBV vaccines have poor cross protection between serotypes, which emphasises the importance of identification and surveillance (Jackwood 2012). IBV variants can appear due to recombination and mutation events in the S1 gene and may occasionally be due to the introduction of virus from other regions (R. C. Jones 2010). Factors that play a significant role in spreading of IBV variants are not fully known, however the trade of poultry products and wild bird migration may have contributed to this dissemination (Sjaak de Wit *et al.*, 2011).

Variants have been detected around the world such as; USA (Jackwood *et al.*, 2010), Latin America (Villarreal *et al.*, 2010), Europe (Worthington *et al.*, 2008), Africa ,(Bouqdaoui *et*

al., 2005), Asia (Lee *et al.,* 2008), Australia (McFarlane and Verma 2008) and in the Middle East (Mahmood *et al.,* 2011). Unlike Arkansas strain of IBV which appears to present only in the USA, 793B and QX strains were widely distributed in many continents (Sjaak de Wit *et al.,* 2011).

On the other hand, D1466 strain has been found in some Western Europe countries but it has not reported from outside Western Europe (Cook *et al.,* 1999). It seems to be that control programme and geographical isolation have helped in a part of preventing the IBV variants from entering other neighbouring countries (Jackwood 2012).

1.4 Transmission

The transmission of IBV happens between an infected flock to another clean one. These flocks could also be a risk to the neighbouring area or further field via migratory birds (Erbeck and McMurray 1998; Hughes *et al.,* 2009; Promkuntod 2016) Hughes et al 2009. The virus is transmitted both directly and indirectly, usually by aerosols created by the discharge from upper respiratory tissues or faeces passed by infected chickens. Moreover, the contaminated items and utensils from staff could be an important way for the spread of infection from one farm to another (Jackwood and de Wit 2013). Recent data also indicates that the virus has the ability for venereal transmission in 54-week old hens (Gallardo *et al.,* 2011).

1.5 Host and breed susceptibility

The primary natural hosts are usually domestic fowl (*Gallus gallus*) and pheasants (*Phasianus* spp.) (Cavanagh *et al.,* 2002); several IBV-like coronaviruses have been detected in domestic and non-domestic avian species, including peafowl, wildfowl, penguins, turkeys, waders pheasants, pigeons, quail, and Amazon parrots (Dea and Tijssen

1989; Circella *et al.,* 2007; Hughes *et al*., 2009; Wu *et al.,* 2016). Antigenic similarity between avian IBV (AIBV) and turkey coronavirus (TCoV) has been reported (Guy 2000).

Different ages and breeds have been shown to be infected by IBVs, but greater severity is evident in young chicks compared with the adults. In the same way, resistance is connected to age, and it rises with increasing age (Crinion and Hofstad 1972a). Some breeds have also been shown to be more resistant. It was a focus of early experiments to demonstrate that resistance to IBV M41, in line C white leghorn chickens, is greater than line 151, although the shedding rate is almost identical (Otsuki *et al.,* 1990; Bumstead 1998). This may be due to the inherited differences in major histocompatibility complex (MHC), such as can be found among three standard chicken haplotypes (Bacon *et al.,* 2004). Moreover, the outcomes indicated that the genetics of B defined haplotypes may be exploited to produce chicks resistant to respiratory pathogens or with more efficient immune responses (Banat *et al.,* 2013). This feature was tested when different breeds exhibited a different mortality rate when IBV inoculation was given alone or in combination with E.coli (Cook *et al.*, 1986). Hence, it seems that IBV resistance is closely related to the breed. This point needs to be taken up in research highlighting the immune mechanisms specific to different types of chickens so it would help to select a breed with inherited resistance in endemic areas to decrease the economic losses from novel IBV strains.

One gap of knowledge in the literature of what remains unknown is how different chicken lines respond in IBV *in vivo* infection. Among this response is the pathogenesis and innate immune responses to not only virulent strains but also to vaccines and connected this with, the local, humoral, and cellular responses. The previous point has been covered in other pathogens but not IBV, amongst types of broilers chicken that are either fast or slow growing breeds. Hence, further studies could find some aspect which relate to field strain

resistance and so decrease losses in IBV infection not only by reducing mortalities but also by retaining body weight during infection.

1.6 IBV entrance and receptors

It has been proven that IBV infects the kidney, trachea and reproductive tract via the interaction of the S1 glycoprotein IBV receptor-binding domain (RBD; present in M41 amino acids 19–69) with receptors on the cell surfaces, namely those of α-2,3-sialic acid (Winter *et al.*, 2006; Shahwan *et al.*, 2013). The RBD in the S1 spike plays a central role in host cell–virus attachment (Babcock *et al.*, 2004; Promkuntod *et al.*, 2014). For this reason, changes in the S1 glycoprotein tend to control the virulence and tissue tropism of the IBV strain (Casais *et al.*, 2003; Thiel 2007). In attenuated Baudette-IBV, another type of receptor beyond sialic acid receptors has been claimed to potentially accommodate the strain's vast host range in cooperation with a putative heparan sulphate (HS) binding site (Madu *et al.*, 2007).

The IBV tissue tropism differences of some strains or variants are still unclear; for example, in the most recent study, the results revealed differences in the susceptibility to infection in oviduct cell culture with the QX but not the nephropathogenic B1648 strain (Mork *et al.*, 2014). Although the attachment was sialic acid dependent, it seems that the variety of the two strains' infection competences in the oviduct is not related to the sugar attachment feature of IBV spike proteins. Moreover, it has been shown in S1 proteins that IBV strains bound with the same effectiveness to oviduct epithelial cells (Mork *et al.*, 2014). Changes in the tissue tropism could be linked to the replicase gene, such as in IBV isolate CK/CH/LGX/130530, and thereby change the viral pathogeneses (Han *et al.*, 2016).

After the virus is attached, structural changes happening in the S1 glycoprotein mediate the membrane fusion activity of the S2 carboxylic acid terminal of the spike glycoprotein (Cavanagh 2007). Then, the virus enter the cell and frees its nucleocapsid in the cytoplasm

of infected cells (Chu *et al*., 2006). The virus replicates in the cytoplasma, causing budding formation and is released.

1.7 Pathogenesis

The respiratory system is the main site infected by IBV. After a short viraemia of 18–36 hours, the virus spreads to different tissues. There are several field isolates and some variants which affect the chickens' digestive, renal and reproductive systems. The pathogenicity scale varies, corresponding to the system involved and virus strain (Cavanagh 2007; Wickramasinghe *et al.*, 2014). This diversity is assumed to be due to the amino acid differences in the primary viral attachment S1 domain of the spike protein (Wickramasinghe *et al*., 2014). Numerous factors have been associated with the clinical consequences in chickens subjected to an infection, such as the age and sex of the birds; type of immune status (including maternally derived Abs [MDAs], immunosuppressive agents and flock vaccine-take status) and virus strain; and environmental conditions, such as cold stress, climate, dust, ammonia and co-infections (Jackwood and de Wit 2013). The body systems that are more affected by some strains than others need to be considered in future research, and it is possible that a variant will emerge that infects some tissues that never been a target in the known classical or variant IBVs.

Typically, the virus produces lesions after replicating in many mucosal cell membranes, including those of the respiratory tract (trachea, Harderian gland, nasal turbinates, air sacs and lungs), reproductive organs (testes, oviduct) and kidneys. The virus also has the potential to proliferate in various cells of the alimentary tract, where it is frequently associated with mild lesions (Raj and Jones 1997b), and it can produce macro- and micro-changes in the small intestine that may be associated with alteration in the S1 structure or mutation (Hauck *et al.*, 2016).

The tropism of some IBVs to infect the kidney more than the tracheal tissues has been the focus of some studies, clarifying the mechanisms of the virus at the infected site and assisting in developing better avoidance strategies to control infection (Cook *et al.*, 2012; Reddy *et al.*, 2015). In the kidney, a unique mechanism probably found, which results in kidney tropism (Reddy *et al.*, 2016).

The severity of lesions in the respiratory tract caused by IBVs is variable; it is influenced by viral virulence, bird age at infection (Jones 1974), genetic susceptibility of the chicken line (Joiner *et al.*, 2007) and maternal or active immunity of the chicken (Awad *et al.*, 2016). Moreover, the severity of IBV infection is also affected by increased susceptibility to primary or secondary respiratory pathogens leading to increased damage from these co-infections. These increased damage from duel viral-bacterial infections have been shown for agents like *E. coli* (Matthijs *et al.*, 2005; Matthijs *et al.*, 2009), *Mycoplasma imitans*, *Mycoplasma gallisepticum* (Ganapathy and Bradbury 1999) and *Mycoplasma synoviae* (Landman and Feberwee 2004). Bacterial or viral complications associated with IBV infection are a wide resource for investigation, and further trials could answer many questions in the context of dual infection with this contagious disease.

Extensive primary viral infection lesions will be exacerbated by secondary bacterial infections, especially in broilers, causing a higher mortality, decreased growth, increased feed conversion ratio, antibiotics consumption and carcass condemnation rates. Evidently, a effective vaccination plan against IBV will help these birds in avoiding clinical IBV disease, and consequently, secondary airsacculitis from *E. coli* infection (Matthijs *et al.*, 2005).

Although some IBV strains are considered nephropathogenic, causing lesions in the kidney under field or experimental conditions (Terregino *et al.*, 2008; Ganapathy *et al.*, 2012),

other strains cause slight or no lesions under the same conditions, leading to the same question about variations in tissue tropism of IBV strains (Khataby *et al.,* 2016).The proventriculus is another tissue targeted during infection by some IBV variants, such as QX and Q1, leading to proventriculitisin infected birds (Yudong *et al.*, 1998; Yu *et al.*, 2001; Ganapathy *et al.*, 2012). However, despite the isolation of this variant from the previous organ, there is no evidence of local proliferation by IBV strains. Moreover, the death percentages were greater than those previously reported during IBV single infection. It seems that these cases were either complicated by another pathogen / factor, which led to elevated mortality. Further experiments are needed to validate proliferation of IBV in the proventriculus after confirmation of the virus's presence in this tissue during embryonic infection by M41 (Abdel-Moneim *et al.*, 2009). In addition, IBV has been isolated from the pancreas of brown/red broilers chickens at 2 weeks of age (Hauck *et al.,* 2016).

Comment [MB1]:

1.8 Diagnosis

The diagnosis of IBV infection is divided between either clinical or laboratory cases and also either during field infection or under experimental conditions. Each test is related to one another; but, some of them, are directly connected together (Figure 1.1).

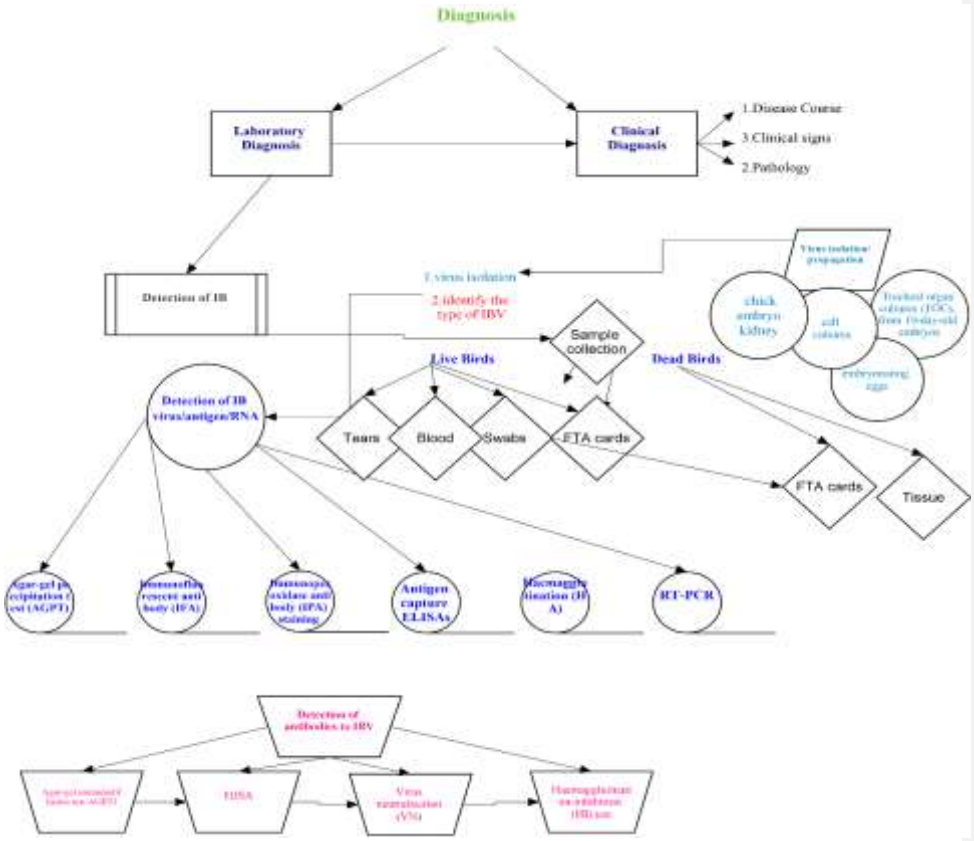

Figure 1.1: Illustration of the process of clinical and laboratory diagnosis of infectious bronchitis virus from suspected clinical samples or during experimental infections.

1.8.1 Clinical signs

IBV has the potential to infect chickens at all ages. In single infections, IBV has a relatively short incubation period in flocks, with reports of 18–48 hours in some broiler chicks (between 2 and 6 weeks of age) (Ignjatovic and Sapats 2000). After 3–5 days post-infection the virus titre reaches the highest point; then, it rapidly decreases, since the virus primarily grows in the upper respiratory tract, subsequently spreading to other, non-respiratory tissues (Jones and Ambali 1987; Lucio and Fabricant 1990). The signs vary according to the chicken breed (Jackwood and de Wit 2013) and the severity of infection

depends on factors such as viral virulence and the bird's age (Ganapathy 2009). Typical clinical signs include tracheal râles, coughing, sneezing and gasping and difficulty breathing, with or without a nasal discharge. In uncomplicated cases, these clinical signs last for only 5–7 days and disappear in 10–14 days (Raj and Jones 1997b). Other signs include a lack of food intake and whitish diarrhoea, watery eyes, swollen sinuses and apparent body weight retardation; the birds may be huddled under heat sources (Jackwood and de Wit 2013).

Secondary infections from other bacterial and viral pathogens, such as *E. coli*, often follow the primary infection, and they can intensify the respiratory signs (Nakamura *et al.*, 1992). In some cases, secondary co-infections with *Mycoolasma synoviae* can result in egg abnormalities (Feberwee *et al.*, 2009). If chronic respiratory disease develops, it may last for several weeks, with mortality ranging between 5% and 25% (Fabricant and Levine 1962). Factors that may affect the severity of respiratory clinical signs include the environmental conditions, breed of chicken, type of housing system, vaccine programme, strain of IBVs and other co-infections (Jackwood and de Wit 2013). For nephropathogenic strains, factors that may increase the mortality rates include a cold environment, a high protein diet and the breed of chicken (Bhattacharjee *et al.*, 1994). Normally, wet litter occurs due to increased water intake (Irvine *et al.*, 2010). Occasionally, after the start of infection, mortality occurs within 4–5 days; the risk of mortality subsides by day 12 (Cumming 1963).

IBV infected layers can have decreased egg production, decreased egg quality (Cavanagh and Naqi 2003) or a loss of shell pigment (Butcher *et al.*, 2011). Some eggs can also have soft, misshapen or rough shells; watery albumin and overall decreased hatchability (Cumming 1969; Ganapathy 2009). In layers, further to the clinical respiratory signs, reduced egg production can range up to 70% (Box *et al.*, 1980; Box and Ellis 1985).

Production levels can also be influenced by factors such as the IBV strain serotype, host immunity and the level of egg production during infection (van Eck 1983). False layers are the result of infection of 2-week-old females, due to the resulting damage to the oviduct (Worthington *et al.*, 2008; Ganapathy 2009; J. J. de Wit *et al.*, 2011).

1.8.2 Pathology

The lesions in the field are often different from those seen under experimental condition. In specific pathogen-free (SPF) chicks, the clinical signs of lesions in the trachea, the kidneys and oviduct are the main findings. In severe cases, the virus spreads from the trachea and causes significant exudation and necrosis in the air sacs (Cavanagh and Naqi 2003). Secondary bacterial infections are sometimes cofactors in more severe disease in the field; however, the viral serotype may also play a role in the disease outcome (Cavanagh and Naqi 2003; Dwars *et al.*, 2009).

Infected chickens may show signs of dehydration, oedema, tracheal congestion, pulmonary congestion, caseation and tracheal plugs (Smith *et al.*, 1985). Air sacs are foamy, especially during the acute infection and then they become cloudy and sometimes contain a yellow caseous exudate (Ziegler *et al.*, 2002). Many publications have reported that IBV infections in young female chickens can lead to cystic oviducts (Crinion and Hofstad 1972b; Chew *et al.*, 1997; Benyeda *et al.*, 2009). In addition, infection during chick development may decrease the weight and length of the oviduct, potentially accompanied by a decreased ovary size (Sevoian and Levine 1957). At early ages, infected females can form a visible, undeveloped oviduct structure, with a blind sac projecting forward from the cloaca (Jones and Jordan 1972). Some infected hens have excessive amounts of exudate in the oviduct (Ganapathy 2009; Awad *et al.*, 2016), and cystic oviducts were found in two female chicks when IS/885/00 was tested in an immunopathogenesis study (Ganapathy 2009; Awad *et al.*, 2016).

The nephropathogenic IBV strains, commonly infect the kidneys, consequently causing increased water intake and diarrhoea , in addition to mortality, such as in the case of nrTW I strain (Xu *et al.,* 2016). Nephropathogenic IBV can produce distended kidneys, enlarged ureters and deposition of urates (Cumming 1963; Ganapathy 2009). In roosters, a main finding is epidydimal lithiasis and a corresponding reduction in fertility was found , both in those that had been vaccinated (Jackson *et al.,* 2006) and those naturally infected with IBV (Villarreal *et al.,* 2007). The signs and severity of these pathological changes in the oviduct and decrease in egg quality and production may vary based on the IBV strains and age of the hen (Cavanagh and Naqi 2003). In uncomplicated infection, the lungs seemed largely unaffected in some experimental studies (Grgic *et al.,* 2008; Boroomand *et al.,* 2012). Further, some IBV variants, such as QX, could be the cause of proventriculitis (Ganapathy *et al.,* 2012).

Gough and his colleagues reported that birds infected with the IBV 793B strain displayed deep pectoral myopathy as a specific gross lesion (Gough *et al.,* 1992). Similarly, this strain was isolated from broiler parent flocks suffering from bilateral myopathy, muscle paleness and swelling, with sometimes prominent fascial haemorrhage in both deep and superficial pectoral muscles (Raj and Jones 1996a). Another type of lesion could be seen under infection with the Q1 strain, represented by an increased thickness to the proventricular mucosa and the presence of a milky fluid when squeezed, where the whole proventriculus appeared swollen and enlarged (Yu *et al.,* 2001). However, another IBV variant has been isolated in cases of proventriculitis (Ganapathy *et al.,* 2012). Characterisation of proventriculitis is important for our increased understanding of IBV's pathogenicity as it has not received much critical attention. Probably, studying IBV Q1 in either SPF or broilers chicks will help clarify this issue and fill in a knowledge gap in this area.

1.8.3 Histopathological and clinical signs in infected chicks

In the trachea, the development of lesions has been divided into three stages, as follows: the degenerative, hyperplastic and recovery stages (Nakamura *et al.*, 1991). Histological changes in early infection include mucosal oedema, de-ciliation and rounded or sloughed epithelial cells (Chen and Itakura 1996). Lymphocyte and heterophil infiltration can be seen within 18 hours of infection. During the regeneration period (approx. 48 hours) after infection, the lamina propria may be infiltrated with lymphoid cells. After 7 days, the establishment of germinal centres can be seen (Siller and Cumming 1974).

In the air sacs, important findings, on the first day of infection are oedema, desquamation of epithelial cells and fibrinous exudates (King and Cavanagh 1991). After this, heterophil infiltration and fibroblast proliferation will increase. Eventually, cuboidal epithelial cell regeneration occurs in the recovery period (Siller and Cumming 1974), which starts at 4–6 days and is completed by 10–20 days (Chen *et al.*, 1996).

In the lungs, lesions comprise epithelial cell desquamation and purulent exudation in the early phase, within 4 days (Ignjatovic and Sapats 2000). A recent study reported histological lesions at 3 days post-infection, with small areas of pneumonia observed in the lungs of IBV-inoculated groups. In addition, pulmonary lesions were limited to the primary bronchi commonly beingdiffuse peribronchial infiltrates of lymphocytes and a few heterophils (Mahdavi *et al.*, 2007).

Comment [MB2]:

In the oviduct, the epithelial cells can undergo decreased cilial height, along with glandular dilatation and infiltration of lymphocytes (Ignjatovic and Sapats 2000), fibroblasts and plasma cells in the lamina propria (Crinion 1972). Although there is some information in the literature regarding infection of the oviduct and testis, the effects of this infection may need to be researched more extensively in the future, with more detailed descriptions and

evaluation of this infection's outcomes. A well-known virulent IBV genotype that has been accompanied by cystic oviduct is QX (Benyeda *et al.,* 2009; J. J. de Wit *et al.,* 2011), which also leads to kidney infection and increased mortality, respiratory signs and complete tracheal ciliostasis (J. J. de Wit *et al.,* 2011).

In the kidney, the conventional histological changes are displayed as interstitial nephritis (Siller 1981). In the case of acute infections, granular degeneration, vacuolation, desquamation of the tubular epithelium and excessive heterophile infiltration are observed (Riddle 1987). Lesions in the medullary tubules are typically prominent (Siller 1981). Necrotic foci may be found during the regeneration stage, and the inflammatory cells shift to plasma cells and lymphocytes in the course of recovery. IBV can cause urolithiasis, showing distended ureters being filled with urates, accompanied by atrophy of the kidneys, and urate calculi, that are usually found in the ureters (Siller and Cumming 1974).

In the chronic stage, interstitial nephritis is seen in the cortex and medulla, accompanied by plasma cell and lymphocyte infiltration (Chen and Itakura 1997). Infiltrating plasma cells have a distended cytoplasm and stain deeply with the periodic acid–Schiff (PAS) stain due to the Russell bodies' immunoglobulin contents (Siller and Cumming 1974).

In the intestinal tract, nonspecific lesions have been reported in the colon (Ambali and Jones 1990). Such lesions include desquamation of the cells of villi tips, congestion and focal infiltration of lymphocytes, macrophages and heterophils. However, one trial attempted to test several IBV strains after passage through the intestinal tract using developed *in vivo* and *in vitro* schemes to reduce not only their presence, but also their effects on the upper respiratory tract. The results revealed that tested strains were causing no lesions in the intestinal tract (Alvarado 2004). Conversely, in a more recent study, the researchers detected IBV-like coronavirus in the intestine and other parts of the digestive

system; the lesions were distended and pale, and their content was wet (Mohammed *et al.*, 2012). Histopathological changes showed cellular infiltration in the lamina propria, blunting of the villi, changes in the crypts and cyst formation. Coronavirus particles were confirmed by negative stain electron microscopy. Transmission electron microscopy of the intestine confirmed coronavirus in the cytoplasm of enterocytes. Moreover, IBV antigens were identified in intestinal epithelial cells, in addition to cells in the pancreas and proventriculus, via immunohistochemistry (IHC) (Hauck *et al.*, 2016).

1.9 Factors affecting the IB diagnosis

1.9.1 Sampling time after infection

Usually, in the case of pathogenic IBVs, the first site for virus replication is the trachea; this is followed by viraemia, and then the virus is transmitted to other organs (Raj and Jones 1997b). It has been reported that the optimal time for virus isolation is 3–5 days after infection, due to the presence of high virus concentrations (Jackwood and de Wit 2013). However, the virus cannot be detected by isolation after two weeks because the titre drops below the detection level (De Wit 2000).

1.9.2 Sampling for IBV detection

Typically, an acute infection in unvaccinated birds will produce a greater amount of IBV in the trachea for viral detection (De Wit 2000). In comparison, in vaccinated layers or breeders with chronic infection, for example, a lesser amount of IBV is present in the trachea (De Wit 2000). For RT-PCR, the trachea and kidney are more suitable organs for the detection of acute infection. In contrast, the caecal tonsil and kidneys are better suited for the detection of a chronic infection (De Wit 2000; Villarreal *et al.*, 2010). In some cases, virus isolation is not always considered as the preferred procedure; therefore, using RT-PCR is preferable for IBV identification. Cloacal or tracheal swabs can be directly processed by RT-PCR for a rapid diagnosis (Cavanagh *et al.*, 1999).

1.9.3 Sample quality

To maintain the virus' viability, clinical tissue samples should be stored immediately at 4°C or frozen at –20°C (especially for isolation). Alternatively, a 50% glycerine solution should be used to keep the samples active for several days (De Wit 2000; Villarreal *et al.*, 2010). If samples (e.g. organs or swabs) are to be stored for a long period, then –80°C is optimal (Ganapathy 2009). Swabs from potentially infected birds should be pooled in sterile media accompanied with antibiotic and anti-fungal compounds (McMartin 1993).

Recently, there has been an increase in sampling by using Flinders Technology Associates (FTA®) sampling cards (Whatman). Birds' swabs or tissue impression smears are applied directly to the FTA card and then dried in air. These cards are made of a cotton-based cellulose paper impregnated with chemicals that bind and stabilize nucleic acids and inactivate proteins . After clinical samples are applied onto FTA cards, their reagents have been shown to inactivate many pathogens and to lyse most cell types. RNA viruses are reported to be relatively stable and FTA cards have been used for the detection of many avian pathogens such as such as Newcastle disease virus, infectious bursal disease, avian Metapneumovirus, Avian Influenza and IBV (Moscoso *et al.*, 2005; Purvis *et al.*, 2006; Awad *et al.*, 2012; Keeler *et al.*, 2012). However, the stability on storage and recovery time of viral RNA demonstrated differences when FTA cards were used for rabies virus detection (Sakai *et al.*, 2015).

Comment [MB3]:

1.9.4 Chicken genetics

Many studies have reported the variation in severity of IBV infection depending on different genetic lines of chickens. In comparative studies of two types of chickens (white leghorn line C and white leghorn line 151) infected with IBV, the genetic differences are produce clear variation in their clinical signs, virus load in tissue and pathological lesions in tracheal epithelial tissue. The results showed that line 151 chickens had more severe

respiratory signs than did line C chickens, although similar amounts of the virus were recovered from the tracheas (Otsuki *et al.,* 1990; Nakamura *et al.,* 1991). Similarly, different mortality rates have been seen among various types of chickens following a challenge of either IBV alone or in combination with *E. coli* (Smith *et al.,* 1985; Bumstead *et al.,* 1989). Purchase *et al.,* (1966) reported that inoculated embryonic chick mortality was different among many types of white leghorn chickens with unmeasured MDAs.

1.9.5 Host immunity during infection

It has been shown that any impairment to the immune system, such as infection with infectious bursal disease (IBD) at an early age, can lead to a lack of immunity, and consequently, increase the potential for a severe IBV infection (Giambrone *et al.,* 1977; Winterfield *et al.,* 1978; Toro *et al.,* 2006). The level of immunity and incubation time against IBV affects the time to detection after a challenge (Rosenberger and Gelb 1978). One study reported that using cyclosporine as a T-cell suppressor had no effect on virus titre or the detection of the M41 strain by the monoclonal Ab (MAb) method following challenge (Raj and Jones 1997b).

1.10 Virus isolation

Now days PCR makes isolation less necessary for identification. In many laboratories, isolation is applied by passages of clinical samples in tracheal organ cultures (TOCs), chicken kidney cell cultures (Otsuki *et al.,* 1979b) or embryonated chick eggs (Darbyshire 1978; Ganapathy 2009). Classically, a field strain will induce visible embryonic changes, consisting of stunted and curled embryos with feather dystrophy (clubbing) and urate deposits in the mesonephros in the second to fourth passage. It should be stated that the isolation of IBV takes a relatively long time and is more expensive compared with RT-PCR. Currently, PCR, immunofluorescence assay (IFA) and ELISA are used for detection. As a result, the isolation performed in combination with other tests can be used to maintain

the sensitivity and save time (De Wit 2000). Hence, alternative techniques, such as IFA, immunoperoxidase assay (IPA), antigenic ELISA and RT-PCR, could be successful for antigen or nucleic acid detection, rather than detection of infectious particles. Furthermore, in a mixed infection, virus isolation (VI) can be hampered by the presence of other viruses (De Wit 2000).

1.10.1 Samples for virus isolation

If samples are to be collected from large suspect flocks, material should be screened from both healthy and infected birds (Jackwood and de Wit 2013). IBV can be isolated from a number of tissues, including the trachea (Naik *et al.,* 2005), lung and kidney (Wang *et al.*, 1997), cloacal swabs (Lucio and Fabricant 1990) and caecal tonsils (Franca *et al.,* 2011). The virus isolation success rate is higher in the early stage of infection (Cook 1984). The reproductive organs in layers can be used for up to 28 days post infection but are more unreliable (Cook 1984). It has been reported that, to confirm IBV infection using antigen detection methods, initial isolation may be achieved in biological systems like embryonated chicks, cell cultures and TOCs. Although IBV does not create specific lesions in any of these systems, they are still useful in laboratories (McMartin 1993) for propagation of the virus. IBV isolation in SPF eggs should take place at 9–10 days of age (Montassier *et al.,* 2008).

In TOCs, the inoculation should be from SPF chicks at 19–20 days (Jones and Hennion 2008). Cultures are only considered negative when allantoic fluid (AF) is collected after third passages (Gelb Jr and Jackwood 2008). Confirmation of an IBV positive culture should ideally be achieved by VN, haemagglutination (HI), immunofluorescence, IHC, electron microscopy or RT-PCR. TOCs are considered the most sensitive approach for serotyping by using VN and virus isolation, especially if egg adaptation from continuous passages is unwanted (Cook *et al.,* 1976b; J. Gelb and M. W Jackwood 1998).

1.10.2 Isolation in embryonated eggs

The clinical signs resulting from IBV infection in embryonated chicks are typical for a viral pathogen and include haemorrhages, embryo dwarfing, curling and death. The signs can differ depending on the serotype and sample origin, and the severity increases with an increasing number of egg passages, such as with serotypes Massachusetts 41, Baudette and Connecticut (Johnson and Newman 1971; Villarreal *et al.*, 2010). It has been shown that the virus reaches its maximum amount after 1–2 days in AF (Darbyshire *et al.*, 1975).

Viral culture in embryonated chick eggs is considered to be the gold standard for isolating IBV from field samples (Cook *et al.*, 1976b). The precise changes from the field strain during the first passage are probably minor. In contrast, by increasing the number of continuous passages, the dwarfing and embryo mortality increase (Clarke *et al.*, 1972). One main disadvantage of this method is that at least three passages may be required for recognising lesions, which prolongs the period to diagnosis (Ashraf M Awad *et al.*, 2014). Furthermore, the IBV may be unviable due to improper storage conditions before egg inoculation. Moreover, dual infections from multiple IBV strains may also hamper replication (Villarreal *et al.*, 2010). Positive virus isolation by this method for non-egg-adapted field strains can take longer than that for egg-adapted strains.

Comment [MB4]:

1.10.3 Virus isolation using cell cultures

Cook *et al.*, (1976a) reported that the first attempt at IBV isolation from infected material in a conventional monolayer cell culture failed. Interestingly, a recent study showed that IBV strains H120, M41, Gray and H52 could be propagated in HeLa cells, with different cytopathic effects (CPEs) (Chen *et al.*, 2007). To obtain optimal viral replication and CPE, IBV should be cell-culture adapted (Gillette 1973). Kidney cell cultures from both chicks and chickens are extremely sensitive to cell adapted IBV strains (Lukert 1966; Otsuki *et al.*, 1979a).

1.10.4 Virus isolation using chicken organ culture

In one report, Cook (1984) demonstrated that TOCs represent a highly successful method for the serotyping, isolation and titration of IBVs, since field strains are not necessarily adapted for growing and inducing nonspecific ciliostasis. TOCs are ideally checked every 24 hours for 4-5 days using inverted optical microscopy to measure ciliary activity, as virus multiplication causes a reduction in the tracheal ring viability (Epiphanio *et al.*, 2002). Due to lower sensitivities, cultures from other tissues are not recommended (Dhinakar Raj and Jones 1996; Bhattacharjee and Jones 1997).

Although TOCs are considered an efficient approach for virus propagation, they are not considered sensitive to field strains that have no tropism or affinity for the respiratory tract. As a result, potential false negative results can arise. In addition, ciliostasis can be induced by other infectious pathogens; therefore, detection of IBV by alternate methods is also required (Villarreal *et al.*, 2010).

1.11 Antigen detection (or IBV identification)

Identification of IBV using antigen detection is usually conducted by egg passage, as field samples need to adapt to chick embryos. This approach is time consuming and requires a massive quantity of the virus. Nevertheless, IBV can be detected from infected field samples directly using IHC, immunofluorescence or immunoperoxidase through IBV-specific MAbs or polyclonal sera (Gelb Jr and Jackwood 2008). AF and tracheal mucus can be used through an agar gel-precipitating (AGP) test, is considered as highly sensitive (Gelb Jr and Jackwood 2008). Antisera are collected from birds infected with IBV or injected with viral proteins, and as a result, Abs against these antigens, such as the virus matrix protein or nucleoprotein, may be present in the collected sera. Biological differences *in vivo* can hamper standardisation and technical performance, therefore, MAbs are used to

supply definitely reproducible products, as they react with IBV antigens with one or more epitopes (Koch *et al.,* 1986).

The main problem with using MAbs to detect IBV antigens results from mutations in the nucleotide sequence (and subsequently, the amino acid sequence) in the epitopes with which the MAbs no longer react. Then according to the MAb used, the viral strain is not IB or not from the same IBV serotype as the Mab is targeted towards (Cavanagh and Davis 1988; Cavanagh *et al.,* 1988; Wainright *et al.,* 1989). This issue can be avoided by using MAbs specifically targeting conserved regions of the virus or by using multi-MAbs (a mixture of MAbs) (De Wit 2000). In this case, the risk is reduced that one alteration or absence of an epitope could lead to a false-negative result in an antiserum-based assay, since multiple MAb epitopes are targeted alongside novel ones (De Wit 2000).

1.11.1 The agar gel precipitating test

The AGP test can be used in the initial stages of infection, although it is considered insensitive for IBV detection, especially when other precipitating antisera tests exist. In addition, AGP can be used for IBV confirmation in inoculated eggs; this method has a greater sensitivity compared with using sera (Gelb *et al.,* 1981).

1.11.2 Immunofluorescence antibody tests

Clarke *et al.,* (1972) used a specific fluorescein stain for allantoic cells from eggs inoculated with IBV. The results showed that this test decreased the time and cost, while showing a higher sensitivity compared with the viral isolation method, which caused dwarfing of chick embryos (Clarke *et al.,* 1972), as well as allowing the detection of IBV from TOCs and cell cultures (De Wit *et al.,* 1995). In addition, immunofluorescence antibody (IFA) testing may be used as group specific when using polyclonal IBV serum or group- specific MAbs (Yagyu and Ohta 1990; De Wit *et al.,* 1995). De Wit *et al.,* (1995)

reported that IFA is sometimes used as a type specific assay using specific MAbs. It has been proven that direct immunofluorescence staining of TOCs can detect an IBV infection (Bhattacharjee *et al.*, 1994), butthe sensitivity of IFA can differ. For example, tissue was taken from chickens in an experiment on SPF chicks, and the results showed the lung to be positive by virus isolation (VI) and also the kidney and trachea for about 2 weeks, whilst IFA identified IBV antigen at 1–7 days in the lung and trachea and for 3–11 days in the kidney (Jones and Jordan 1972). So the sensitivity can be low when used on infected field tissue, and this is potentially lower than VI (De Wit 2000). Many researchers have reported the difficulty in reading the fluorescein due to non-specific reactions (Yagyu and Ohta 1990; De Wit *et al.*, 1995). This issue is more significant in field samples than with samples from experimental birds, as additional factors like *E. coli* infection, ammonia and dust can lead to epithelial damage (De Wit 2000). IBV can also be detected by using IFA from TOCs (Bhattacharjee *et al.*, 1994), embryonated eggs (Pensaert and Lambrechts 1994) and cell cultures (Wainright *et al.*, 1989).

Comment [MB5]:

Two methods can be used to increase the specificity of IFA, namely decreasing the time of storage at low temperatures between sampling and fixation (Braune and Gentry 1965) and using MAbs rather than hyper-immune anti-IBV serum (Yagyu and Ohta 1990; De Wit *et al.*, 1995). Although VI is more sensitive than IFA overall, especially at the end of infection (in some research, the detection end point has lasted for 14 days) (Ambali and Jones 1990), there is also the potential for IFA to have a higher sensitivity than VI does (Hawkes *et al.*, 1983; De Wit *et al.*, 1995). It is necessary for the virus to be infectious and viable when VI is targeted; however, this is not the case with IFA, since it only needs viral proteins or an *in vitro* probability of some viral replication (Hawkes *et al.*, 1983; Yagyu and Ohta 1990). In addition, when mixed IBV infections are present, there is a variation in the quantity and speed of viral multiplication between the strains that can lead to hampered

proliferation for either one or both. Therefore, a virus with limited antigen production may be undetected with this approach (Droual and Woolcock 1994; Cavanagh *et al.*, 1999).

1.11.3 Immunoperoxidase assay (IPA)

Like IFA, IPA is a staining technique for Abs. After fixation (e.g. with acetone (Naqi 1990)) and embedding of the tissue section or organs, a substrate and chromogen are added. An anti-IBV-specific peroxidase-labelled conjugate connects with the IBV and dyes the antigen sample for detection.

Compared with IFA, IPA has the advantages of evaluating antigen-connected cells in addition to general tissue morphology. Assessment of the slides can be achieved with light microscopy, and the slides can be stored for prolonged periods. These features differ from IFA, where staining can fade if the samples are not kept frozen and away from light exposure (De Wit 2000). However, IPA requires a few days to carry out, and it is sensitive to background staining (De Wit 2000). Removing of endogenous peroxidase is preferable during the IPA procedure (De Wit 2000). A previous study detected IBV from chorioallantoic membranes (CAMs) in inoculated eggs using IPA within 15 hours post-infection, with a detection limit of 106.2 EID50 (50% egg infective dose)(Naqi 1990). Recent study results have revealed that IPA had 83% and 84% sensitivity and specificity, respectively, in contrast to RT-PCR which was positive 31%, when IBV 793B was detected from different tissues (Shamsaddini-Bafti *et al.*, 2014).

1.11.4 Electron microscopy

Berry *et al.*, (1964) reported that IBV has a distinct morphological structure which bear projections on their surfaces in negatively stained preparations, and virus characterisation can be carried out according to these features. However, although morphological differences among different IBV strains have been reported (Harkness and Bracewell

1974)such as pleomorphism in the virion shape, absence or presence of a corona and differences in projection shape, a high level of experience is required to interpret IBV particles observed in this manner.

1.11.5 Antigenic ELISA

Antigenic ELISAs are used to detect IBV using IBV-MAb bound specific micro-titre plates (De Wit 2000). Many studies have reported that antigenic ELISAs represent an accurate and precise method for IBV detection compared to AF (Boots *et al.*, 1992; Naqi *et al.*, 1993; Ignjatovic and Ashton 1996) and the detection limitation is around 106 EID50 (Yagyu and Ohta 1987; Nagano *et al.*, 1990), 103 plaque-forming units (PFU) or 100–1000 median ciliostasis doses (CD50) (Ignjatovic and Ashton 1996). In one report, the author suggested that this quantity of virus is essential for the detection of IBV, further highlighting the relative insensitivity of the test (De Wit 2000).

1.12 Indirect diagnosis

Several indirect assays are employed to detect specific Abs against IBV, including the VN test (Witter 1962), HI test, agar gel immunodiffusion test (Dawson and Gough 1971) and ELISA (Mockett and Darbyshire 1981), which are considered mainly for detection the Ab initiated after IB infection or vaccination. In this way, an Ab that arises after the infection or vaccination will be detected and measured; these Abs are consider specific for IBV. In this technique, two samples should be collected, one at the start of infection, and the other after 4 weeks (Villarreal *et al.*, 2010).

De Wit (2000) described several factors that are considered important which can interfere with specific Ab detection, such as the time of infection, age of the birds, immunity at the time of infection, maternal Ab level, vaccination status and number of samples.

1.12.1 Virus neutralisation (VN)

VN is considered serotype specific and exhibits high specificity; detection occurs when the Ab interacts with S1 protein epitopes and prevent cell adhesion (Gelb and Killian 1987; de Wit *et al.,* 1997). It can be carried out in cell cultures, TOCs or chick embryos for Ab detection (De Wit 2000). Hesselink (1991) reported two approaches to VN testing, alpha and beta. In the alpha method, diluted viruses are used in different concentrations with a fixed serum concentration. For beta testing, various serum concentrations are used with a fixed volume of the virus. VN is a serotype-specific tool for identifying the primary and secondary phases of the immune responses, and the results reflect that the test Ab shows good serotyping precision *in vitro*. This is because IBV-neutralising Ab is found to the S1 protein (Karaca *et al.,* 1992; Ignjatovic and Galli 1994). But it has been shown that there is a strong correlation between the total inoculum volume and specificity, as neutralisation is far higher from a single inoculation (Marquardt *et al.,* 1981; Gelb and Killian 1987).

Ladman *et al.,* (2006) showed that VN tests using Archetti and Horsfall's (Archetti and Horsfall 1950) method could be employed to calculate the antigenic association values among strains using chick embryos. Moreover, Dolz *et al.,* (2006) stated that the VN results showed a slight antigenic correlation (less than 35%) among some IBV reference strains, such as M41 and IT02.

1.12.2 The haemagglutination inhibition test (HI)

Like in the VN test, the HI test identifies Abs to the IBV S1 spike glycoprotein, and the test requires 7–14 days from when the samples are taken to confirm Ab detection (de Wit *et al.,* 1997). For this, the IBV test sample needs to be treated with neuraminidase to agglutinate chicken red blood cells. The HI test is strain and serotype specific, especially after the first inoculation (De Wit 2000). A titre of below 32 is considered negative.

1.12.3 ELISA

ELISA testing is an essential, classical serological method for Ab diagnosis; the assay provides evidence of infection or vaccination, depending on the time of sampling and seroconversion. An earlier study developed and used a MAb-blocking ELISA; this was compared with a conventional, indirect ELISA. In this experiment, chickens were inoculated with three strains, namely Connecticut, Massachusetts and Arkansas(Karaca and Naqi 1993). The results showed good correlation between VN and ELISA.

1.12.4 IBV genomic detection

Many trials have been reported that are being used for detection of a part of the IBV genome (Cavanagh *et al.,* 1999) or the entire genomic RNA (Xue *et al.,* 2012) using different primers. The routine procedure involves three steps. The first step is proliferation of the virus from clinical samples *in vitro* using TOCs or embryonated eggs. In the next step, RT-PCR is employed to translate the genome alongside using polymerase to proliferate the cDNA numerous times. Ultimately, one of the following approaches is used: sequencing (Cavanagh *et al.,* 1986), RFLP (Kwon et al.. 1993) or hybridisation (Jackwood *et al.,* 1992). Typically, the S1 gene is the most significant part to be detected via the specific primers, which are designed from the hypervariable region in the S1 gene. These primers are considered specific for different IBV strains, such as Conn, JMK, Delaware, Mass, Ark, California C/633/85 (Keeler *et al.,* 1998), 793B, D1466 and D274 (Cavanagh *et al.,* 1999). However, universal primers that have been tested before, which also correlate to the S1 gene, are being employed in many IBV serotypes (Worthington *et al.,* 2008). In contrast, the results when using RT-PCR with clinical samples and tissues directly are often insufficiently sensitive (De Wit 2000). Therefore, TOCs and chick embryo are usually used for the proliferation of IBV before RT-PCR (De Wit, 2000), or in some cases, they may be utilised directly from infected birds (Roberts and Chousalkar 2008). Some

factors have been mentioned as affecting the RT-PCR reaction which include the amount of ions in the sample (e.g. Mn 2+) and the use of a control to check for cross-contamination (Jackwood *et al.*, 1997). An important factor is using specific primers to detect IBV strains by RT-PCR (Zwaagstra *et al.*, 1992).

There are many types of RT-PCR, including universal (Worthington *et al.*, 2008) and unique types, which are used to differentiate vaccine strains from variants, such as the Massachusetts vaccine and Brazilian field genotypes of AIBV (Fraga *et al.*, 2016). Further types of RT-PCR are duplex RT-PCR (Saba Shirvan and Mardani 2014) and GeXP-multiplex PCR assay, which was utilised to differentiate nine avian pathogens at the same time (Xie *et al.*, 2014).

At the same time, qRT-PCR has also been used in the detection of IBV (Marandino *et al.*, 2016), or qRT-PCR data with the use of universal and type-specific primers (Roh *et al.*, 2014). Moreover, real-time detection is employed, as in the SYBR Green I real-time RT-PCR (Fellahi *et al.*, 2016) and Taq Man probe methods (Callison *et al.*, 2006), which are considered more sensitive than conventional RT-PCR. Using the PCR products from RT-PCR, with sequencing and phylogenetic analysis exploration of the evolution of novel emergent IBV genotype or strain is possible.

More recently, there has been successful high-resolution melt (HRM) curve analysis of PCR products, which was found to be sensitive and rapid in detecting variants and able to differentiate between field and vaccine IBVs (Hewson *et al.*, 2010). At present, the efficiency of next-generation sequencing (NGS) has been established, analysing the full-length genome sequence in a short time frame (Reddy *et al.*, 2015). Using one approach will be preferable over another, depending on the assay's cost, purpose and sensitivity and specificity.

1.13 Strain classification

To date, there is no consensus on a classification system for IBV strains. The favoured classification system correlates with the aims of either vaccination or epidemiological surveillance, as well as cost, experience and the availability of the technique (De Wit 2000).

To establish a proper protection scheme to control IBV, typing of field strains is necessary. It is well known that IBV is classically characterised by forming new serotypes and genotypes, since there is either a high mutation rate or during recombination of amino acids (Cavanagh *et al.,* 1992; Jia *et al.,* 1995). Serotypes or genotypes result from an alteration in the amino acid level in S1 gene (De Wit 2000). The detailed classification of IBV was reviewed by de Wit (2000), who found that IBV strains are classified by functional and non-functional groups. The first group includes strains classified by the biological activity of the virus, leading to specific pathotypes, protectotypes and serotypes. In contrast, the non-functional group includes strains related to the viral genome. The pathotype is defined by the criteria of tissue tropism and major lesions, which are demonstrated by an IBV strain in natural hosts (Cavanagh 2005). However, major issues occur with classifying IBV strains based on the different manifestations of virulence due to frequent strain mutation and the lack of test standardisation (De Wit 2000).

Serotypes are categorised according to VN after IBV isolation in embryonated eggs, TOCs or cell culture. In contrast, protectotypes depend on cross-immunisation study for typing. Moreover, genotyping is achieved by genetic characterisation using RT-PCR and RFLP for cleavage site analysis and sequencing (Callison *et al.,* 2006).

1.13.1 Serotyping

Traditionally, the HI test (King and Hopkins 1984) and VN in TOCs (Darbyshire *et al.,* 1979), chick embryos (Dawson and Gough 1971) and cell cultures (Hopkins 1974) are

used for serotyping. In this typing system, two strains (e.g. a and b) may be considered to belong to the same serotype if two-way heterologous neutralisation titres (antiserum A with virus B) varies less than 20-fold from homologous titres (virus B with antiserum B, virus A with antiserum A) in both directions (Hesselink 1991). However, not all IBV strains spontaneously haemagglutinate red blood cells, and they ought to be treated with neuraminidase for HI (Ruano *et al.,* 2000).

The researchers used fluorescent foci for serotyping (Csermelyi *et al.,* 1988). Furthermore, ELISA and MAbs have also been used for serotyping and distinguishing IBV strains (Ignjatovic and McWaters 1991). Nevertheless, this method has some drawbacks, such as poor availability of MAbs and it is essential to produce new MAbs with each new variant (Karaca *et al.,* 1992). Serotyping becomes impractical, since more and more new IBV strains are being detected, and these must be distinguished from those already present to carry out a neutralisation test. Furthermore, the antibody must be prepared in SPF chickens.

1.13. 2 Genotyping

Genotyping is done using specific or universal primers, with differentiation carried out by nucleotide sequence analysis. A drawback of this classification method is that the detection may be restricted by the primers used. Although universal oligonucleotides are adequate for the detection of most known IBV strains, emerging variants or some strains in a mixed sample may be missed (Ganapathy 2009). In this type of classification, strains are typed according to their genetic characterisation (usually from a part of the genome). In other words, IBV strains may be typed according to the genetic characterisation of the S1 subunit, especially the S1 hypervariable region (Cavanagh 2005). Genotyping is not the preferred approach for independent use in classification. Therefore, a basic approach, such as the VN or HI serotyping, may be supported by *in vivo* experiments for IBV classification accompanied by genotyping (De Wit 2000).

Field strains that were classified by the VN or HI serotyping method have been replaced by genotyping (Jackwood and de Wit 2013). This is considered the most common method (Lee and Jackwood 2000). Briefly, RT-PCR is carried out, followed by either sequencing or RFLP through determination of the enzyme cleavage site (Lin *et al.,* 1991). It has been reported that genomic data are objective and supply important evidence to be used in epidemiological research (Sjaak de Wit *et al.,* 2011). However, many researchers have demonstrated conflicting results between serotyping and genotyping (Capua *et al.,* 1999). To summarise, De Wit and colleagues (2011) stated "genotyping is an excellent tool for epidemiological studies, and is a convenient, practical tool for typing that can be used best as a means of screening to select potentially relevant strains. In situations where there is suspicion in the field that the genotype of recent isolates does not provide accurate information about the actual antigenic nature of these IBV isolates, then conventional testing (serotyping) and especially *in vivo* protection studies are required" (Sjaak de Wit *et al.,* 2011).

1.13.3 Protectotyping

Classification in the protectotype category involves decreasing the number of serotypes to protectotypes, which are important in vaccine programmes in the field. Protectotyping is beneficial and applicable; if two strains have cross-protection, this means they belong to the same protectotype (De Wit 2000). Few strains, such as M41, can be considered protectotypes which provide cross-protection against other strains. Serotypes that belong to the same protectotype have a common epitope responsible for cross-immunity. Strains that are not linked serologically may still protect each other, such as the H120 and T strain, which originated from Australia (Darbyshire 1985).

To determine the protectotype, a cross-immunisation challenge study may be more beneficial; moreover, it may tend to reduce the number of protectotypes compared with

serotypes, since the immune responses will be missing (McMartin 1993). However, this type of research is expensive, cumbersome, and require a large amount of birds, effort and technical equipment for isolation. The researchers found an alternative way to apply cross-immunisation testing using TOCs or ovary organ cultures from vaccinated hens; this was followed by using *in vitro* heterologous or homologous inoculation strains to evaluate the cross-immunity (Dhinakar Raj and Jones 1996).

Comment [MB6]:

1.14 Immunity

Collectively, the natural, local and cellular and humoral immune systems play an essential role in the control of IBV infection. The Hadrian glands (HGs), considered the main eye-associated lymphoid tissues draining secretions in the respiratory tract, and the conjunctiva of the lower eyelid (CALT), are involved in the local immunity in the eye. HG secretions (secretory immunoglobulins [sIgs]) protect the upper respiratory tract (RT) (Gurjar *et al.*, 2013). All the local immunoglobulins (IgM, IgA and IgY [IgG] in the lachrymal fluid are produced by the HGs, which provide local protection in the upper RT via the immunoglobulins. After IBV attenuated vaccine application, proper correlates of protection have been proposed, represented by IgG and IgA levels in tears (Okino *et al.*, 2013). Recently, it has been reported that simultaneous application with Ma5 and 4/91 vaccine strains induces high levels of immunoglobulins (IgA and IgY) in the URT washings and high level of CD8+ T cells in the HGs (Śmiałek *et al.*, 2016; Zhang *et al.*, 2017). The levels of the different immunoglobulins (IgY, IgA and IgM) were reported to be different in the chicken line (Nakamura *et al.*, 1991).

In the trachea, the ciliated epithelium has cells that produce mucous goblet cells. Lymphoid follicles and scattered lymphoid cells consisting of antigen-presenting cells (APCs), CD4+ and CD8+ cells are found in the lamina propria. Hyperplasia of the alveolar mucous glands and goblet cells is considered the first step in triggering the innate immunity after IBV

infection (Nakamura *et al.,* 1991). In addition, after vaccination, extensive lymphocyte infiltration and large numbers of B and T cells and plasma cells have been reported, as the trachea is extremely reactive to IBV (Matthijs *et al.,* 2009).

The innate immune system consists of different cell types, including natural killer (NK) cells, macrophages, granulocytes and dendritic cells (DCs) and proteins (antimicrobial peptides, complement). Ultimately, T and B cells, which are considered adaptive immune system cells, may be stimulated by APCs to help in countering infection and supporting recovery (Chhabra *et al.,* 2015a). APCs, including macrophages and DCs, play a central role as regulators of the adaptive immune responses by interacting with T cells (CD4+ and CD8+) and B cells, whereas NK cells elicit antiviral reactions by killing the virus-infected cells (Figure 1.2).

Pathogens that cross the physical barriers encounter macrophages, which are the main defence line in the infected tissue, and they are massively distributed in that tissue. With DCs, they produce cytokines and chemokines, which trigger the local inflammatory phases (Hawkes *et al.,* 1983). These inflammatory responses in the early stage of IBV infection in the respiratory tract and/or other organs are induced by the high influx of proinflammatory cytokines, which are involved in the activation of innate immune responses by recruiting mononuclear leukocytes and polymorphs to the primary site of viral infection. , The overexpression of these cytokines may be associated with tissue destruction (Kameka *et al.,* 2014).

The recognition of IBV always results in the activation of innate cells, such as macrophages, heterophils and DCs, by Toll-like receptors (TLRs); following this, the downstream immunomodulatory cascade is initiated. Conserved structures in the infectious agents are rapidly recognised by these cells through pattern recognition receptors (PRRs).

In chickens, there are three crucial PRRs, TLRs, retinoic acid-inducible gene (RIG-I) -regulatory induce gene -like receptors (RLRs), and NOD-like receptors NLRs; (Chhabra *et al.*, 2015a). Recently, in-depth studies have been performed on PRRs and the immunological parameters that accompany their expression (Okino *et al.*, 2013; Kint *et al.*, 2015). Researchers have found that TLR3 induces INF-I production through double-stranded RNA viral sensing (Kint *et al.*, 2015). Then, the produced IFNs actively stimulate adjacent cells to express high antiviral proteins, stimulating NK and macrophage cells to elicit antiviral responses.

When antigen-specific immunity is triggered, a type II IFN (IFN-γ) is produced by DCs, NKT, NK cells, CD8+ effector cytotoxic T lymphocytes and Th1 CD4+ T cells. Type II IFN increases the expression of major histocompatibility complex (MHC) class I molecules in healthy cells, and antigen presentation on macrophages promotes the NK cell activity and leukocyte adhesion and binding required for migration. It has been asserted that cytotoxic T cells (CT), which are expressed in the cellular mediated immune response (CMI), are essential for limiting an IBV infection during the early stage. The MHC controls the CTL action, and it is associated with CD4+ and CD8+ cells (Joiner *et al.*, 2007). After that, the humoral immunity starts through B cells, which are stimulated by T-dependent antigens (help from Th cells). APCs with class II MHC proteins process and present antigen to CD4+ T cells (helper cells). Helper T cell become activated. Activated T cell secreted cytokines that in turn activate B cells. B cell differentiates into effector and memory (plasma) cells and produce antibodies (Figure 2.2).

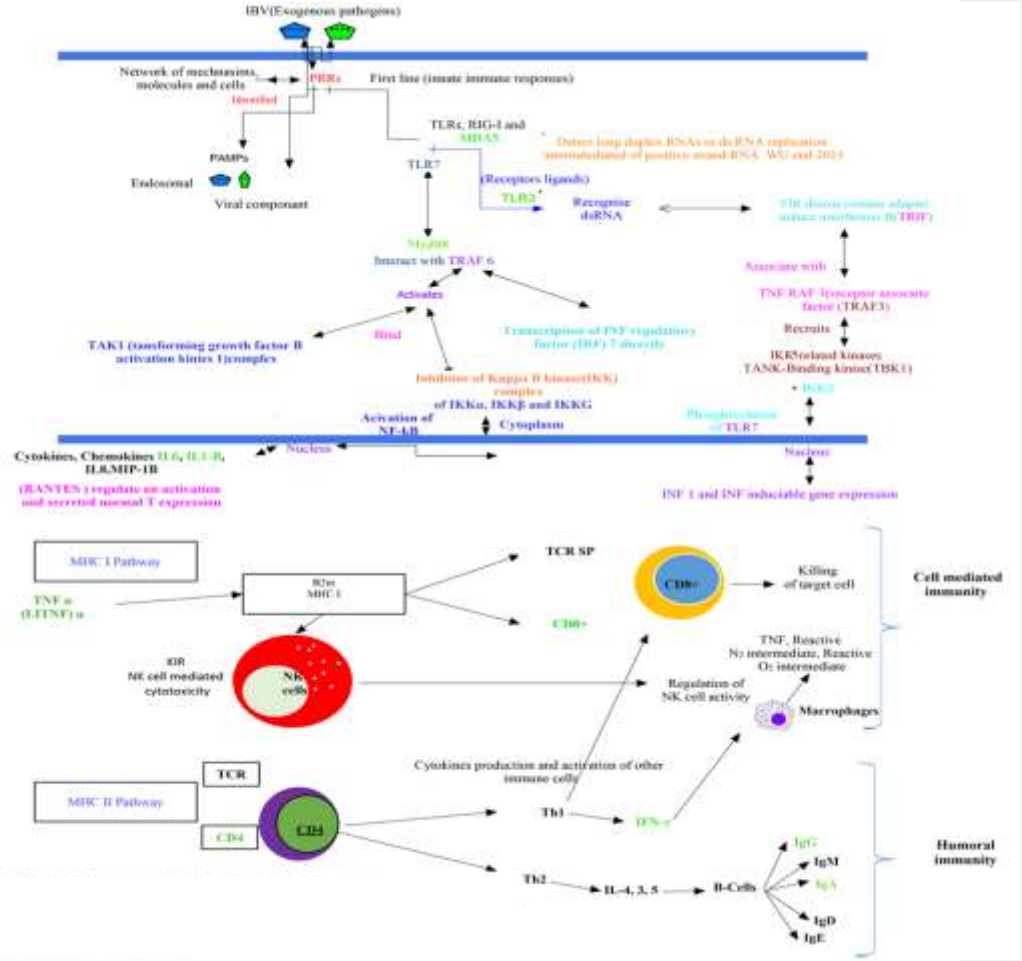

Figure 1.2. Model of innate immune recognition of virus in chickens. The plasma membrane receptor of TLR3 recognize PAMPs. Viral recognition relies on intracellular vesicles of PRR, whose ligands are dsRNA derived from viruses or virus-infected cells(TLR3), ssRNA derived from RNA viruses (TLR7), short 5′ppp dsRNA (RIG-I), and long dsRNA (MDA5). TLR3, TLR7 localize mainly in the ER in the steady state and traffic to the endosome, where they engage with their ligands. The recognition triggers the downstream signal transduction to activate NF-κB or IRF3/7, finally induces interferon and inflammatory cytokine production. Model of T cell differential and the cellular and humoral immune responses after activation of MHC pathways and the subsequent

immunomodulatory responses (green colour indicated the target genes or cells involve in this study).

1.15 Study aims

To improve diagnosis of IBV, in particular through use of molecular techniques. In addition, to enhance our understanding on the molecular pathogenesis including early immune responses following infection of virulent or vaccine IBV strains in *in vivo* and *in vitro* infection models. These aims were achieved through the following specific objectives:-

- To design and evaluate a universal RT-PCR for amplification and sequencing the full S1 for six strains (M41, D274, 793B, IS/885/00, IS/1494/06, and Q1) stored onto FTA cards at 4 ^{o}C, 40 ^{o}C, and 24 ^{o}C (Chapter 3).
- To investigate the pathogenicity and host innate immune responses of variant IBV Q1 in SPF chicks (Chapter 4).
- To compare the pathogenicity and early host innate immune responses to variant IBV Q1 in two broiler lines, Line-A and Line-B, with differing growth rates (Chapter 5).
- To establish an *in vitro* model for assessment IBV vaccine strains in tracheal organ cultures, which will support 3Rs (replacement, refinement and reduction) requirements (Chapter 6).
- To validate the chapter 6 results *in vivo*, to compare the pathobiology and host immune responses following administration of single live vaccines in day-old SPF chicks (Chapter7).

Chapter 2: Materials and methods

2.1 SPF eggs for *in vivo* and *in vitro* experiments

All SPF embryonated chicken eggs that were used in tracheal organ culture (TOC) preparation, virus titration, propagation and virus isolation in all chapters were obtained from a commercial source[1]. Eggs were incubated at 37°C with 55% humidity. The eggs originated from a parental flock free from chicken pathogens, including IBV.

2.2 Tracheal organ culture (TOC)

All TOCs were prepared from embryonated SPF eggs at 18-19 day post incubation. All stages of preparation were under sterile conditions. First, the eggs were wiped with 70% alcohol, viable chicks were humanly euthanized and the tracheas were removed using sterile forceps and scissors. After removing any attached tissues, the trachea were placed in pre-warmed TOC culture medium (Appendix I).

A tissue chopper[2] was used to obtain 0.6 mm-thick rings from the removed tracheas. A single ring was placed into a sterile tube[3] containing 600µl of pre-warmed TOC media. Tubes were incubated in a rotating incubator[4] (8 revolutions per minute's (rpm)) at 37°C. Ciliary activity was checked after 24 hours by an inverted microscope under low power (100 x magnification). Only rings with 100% ciliary activity were included for *in vitro* work.

[1] Novartis, Liverpool, UK

[2] McILWAIN tissue chopper

[3] Nunclon™ culture tubes, sterile, Thermo Scientific, UK

[4] Lab Thermal Equipment, Greenfield, NR OLDHAM

2.3 Specific pathogen free (SPF) and broiler chicks for *in vivo* studies

Embryonated SPF eggs were incubated at 37°C (Figure 2.1A). After checking for fertility on day 18, eggs were moved to a static hatching tray for hatching by day 21(Figure 2.1B). Day-old broiler chicks were purchased from a nearby commercial hatchery[5]. Chicks were kept in an isolation unit (University of Liverpool) throughout all *in vivo* experiments and reared on deep litter with water and feed (free of antibiotics) were provided *ad-libitum*. All experimental procedures were undertaken following approval by the University of Liverpool ethical review committee and according to UK legislation on the use of animals for experiments.

[5] Frodsham Hatchery, Frodsham, UK

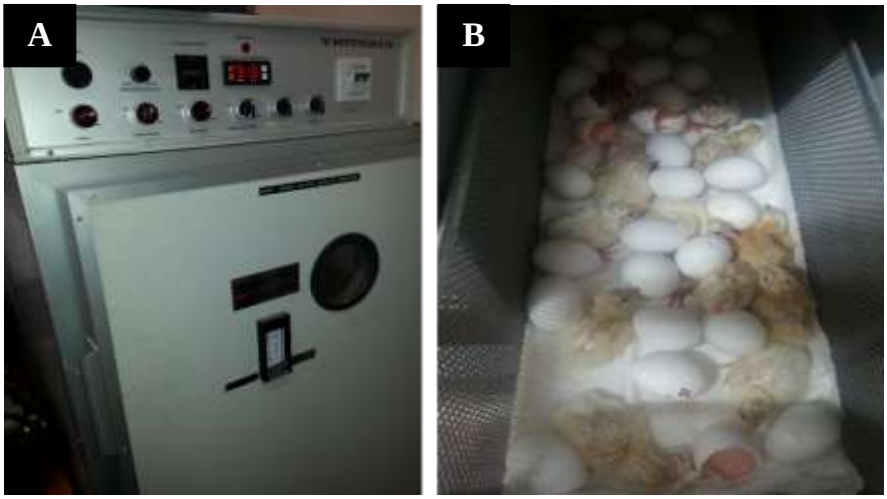

Figure 2.1. The process of hatching from SPF embryonated chicken eggs.(A) Eggs were incubated at 37 °C for 21 days at 55% humidity. (B) One day old hatched chicks for experiments.

2.4 Chick welfare and management

All work was conducted in accordance with UK legislation governing experimental animals and was approved by the University of Liverpool ethical review process. All animals were checked twice (minimum) daily to ensure their health and welfare. All chicks were housed securely with ventilation and temperature adjusted according to age. Chicks were reared on wood shaving litter with food and water supplied *ad libitum* (Figure 2.2 A). Both feed and water was free of preventive or therapeutic medications or herbal products. Individual groups were kept in separate isolation rooms to prevent cross-contamination. Personal protection equipment (PPE) was used at all times during postmortem valuation and tissue collection, including disposable facemasks, gowns, and gloves (Figure 2.2 B).

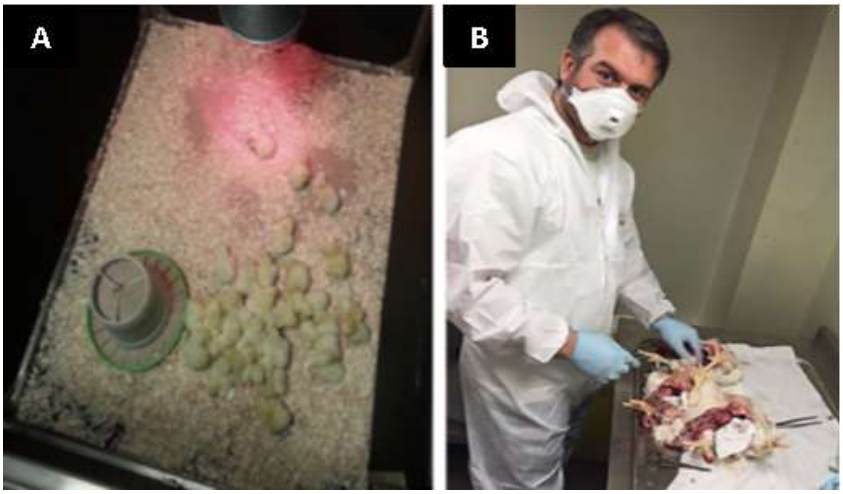

Figure 2.2. Chicks management and birds necropsy.

(A) Chicks at day one old rearing in deep litter and chicks were given *ad libitum* access to food and water. (B) Appropriate personal protection equipment (PPE) at necropsy.

2.5 Virulent infectious bronchitis virus propagation

Allantoic fluid containing infectious bronchitis viruses was checked for contamination of other avian viruses such as Newcastle Disease virus, avian metapneumovirus, avian influenza, infectious laryngotracheitis virus, infectious bursal disease virus and adenoviruses by PCR, and bacteria such as *Escherichia coli* and *Mycoplasma spp* by culture prior to use (Awad 2014). All IBV-rich allantoic fluid was aliquoted and stored at -70 °C after being titrated in TOCs according to the Reed-Muench method (Reed and Muench 1938).

2.6 Vaccine virus propagation

All commercial vaccines were reconstituted in 1mL of TOC media (Sterile distilled water, 900mL; 10x MEM+ EBSS + L glutamine 100mL; 7.5% NaHCO3 20mL; Penicillin /Streptomycin 4mL), except for QX2 (Chapter 6 and 7), which was reconstituted in 3 ml.

For each vaccine, 0.1 mL of reconstituted vaccine was separately inoculated in the allantoic cavity of 10 SPF eggs and incubated for 48h at 37°C. Eggs were monitored daily by candling and non-specific dead eggs (within the first 24 hours) were discarded. After 48 hours, eggs were kept at 4^{o}C for 24 hours, and allantoic fluid was collected from each egg. Collected allantoic fluid was centrifuged, pooled, aliquoted and stored at -70°C until used. Representative aliquots were titrated in ECE or TOC before used in either *in vivo* or *in vitro* experiments.

2.7 Swabs and blood sampling

For *in vivo* experiments (chapters 4, 5, and 7), oropharyngeal and cloacal swabs were collected along with blood samples. Swabs were subjected for RT-PCR to detect IBV and were investigated for genetic differences compared to the inoculum used. Blood was collected in 1.5ml Eppendorf tubes and left overnight in a slanted position to separate serum.

2.7.1 Swab samples

Dry swabs[6] were used to sample the cloaca and oropharyngeal cavity. After sampling, swabs from each group were pooled and dipped into 1.5 ml of TOC media. The media underwent 2x freeze-thawing and stored at -70^{o}C until required for RNA extraction.

[6] Medical Wire and Equipment (MWE)

2.7.2 Serum

Blood was collected from the brachial wing vein using a sterile needle, and placed in labelled bijou tubes without coagulant. In order to obtain serum separation, tubes were kept at a 45^{o} angle. Samples were centrifuged, sera collected and stored at -20 ^{o}C until needed.

2.8 Clinical and pathological assessments

2.8.1 Clinical signs

Clinical signs were recorded daily according to a previously published scoring system (Grgic *et al.*, 2008), using the following criteria: 0 = no signs; 1 = mild signs; and 2 = moderate signs. 3 = severe. Mild gasping, coughing, râles or depression were considered as mild signs, whereas severe gasping, coughing, or depression, or both, accompanied by ruffled feathers were recorded as severe signs. (Grgic *et al.*, 2008). If the chicks exceeding the severity limit were removed from the experiment in accordance with the project license.

2.8.2 Pathological changes

For Chapter 4 and 5, the trachea, kidney and proventriculus were observed following culling and dissection, with gross lesions recorded as described below. (Mahgoub *et al.*, 2010) (Table 2.1).

Table 2.1 Scoring system used for gross lesion assesment following IBV infection

Tissue	Lesions	Score
Trachea	No lesion	0
	Congestion	1
	Mucoid exudate	2
	Caseous plug or airsacculitis, perihepatitis and pericarditis	3
Kidney	No lesion	0
	Swelling and pale	1
	Swelling with visible urate	2
	Large swelling, pale with tubules and ureters distended with urates	3

3.9 Tissue samples for isolation and RT- PCR

All sampled tissues were collected using sterile instruments. Tissues for quantitative real time reverse transcription polymerase chain reaction (qRT-PCR) were collected in RNA later[7] to preserve the integrity of tissue RNA. For virus isolation, the sampled tissues were ground aseptically using a sterile pestle and mortar with sterile sand and TOC media (Appendix I). Samples were stored at -70 ^{o}C until required. Samples were subjected to RNA extraction and RT-PCR to detect the partial S1 gene.

3.10 Virus isolation in embryonated SPF eggs

Isolation and propagation from previously ground tissues was achieved in fertile SPF eggs at 9-10 days old. After candling eggs to ensure viability, 200µl of ground tissue supernatant was injected into the allantoic cavity aseptically and incubated at 37 ^{o}C. Eggs were monitored daily and dead embryos within 24 h were removed. During the first and

[7] Qiagen,Uk

second passages the allantoic fluid (AF) was collected after 48 hours. For the third passage, allantoic fluid was collected after 5 dpi (Figure 2.3). A positive IBV isolation was confirmed by RT- PCR and were sent for commercial sequencing using the forward primer SX3+ (Source BioScience Ltd, Nottingham, UK).

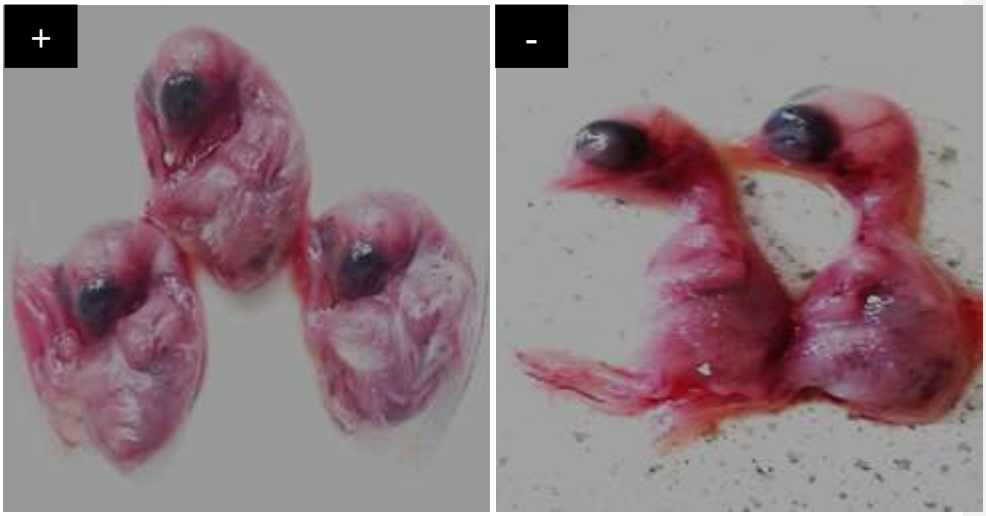

Figure 2.3. Inoculated eggs at 5 dpi. (+) Dwarfism, curling, haemorrhages the characteristic effects caused by IBV in chicken embryonated eggs, (-) negative control

2.11 Histology

Histological changes were scored as follows: 0 = no change, 1= mild, 2 = moderate, 3 = severe (Chen and Itakura 1996; Chhabra 2016), Trachea and kidney samples were processed for histological examination after fixing in 10% buffered formalin (Appendix I) then embedded in paraffin and sectioned at 5 µm thickness. Slides were stained with Haematoxylin and Eosin (H&E) (Chhabra 2016). Proventriculus samples embedded in Optimal cutting temperature compound (OCT) were cut at 5 µm sections using a pre-

cooled cryostat and mounted on glass slides. Slides were washed with 95% ethanol three times and then washed with tap water. The Haematoxylin stain was then applied for 30 seconds followed by another wash with 95% ethanol. Eosin stain was then applied followed by a final wash with 95% ethanol and 100% ethanol (Appendix I). Slides were then washed in xylene and a coverslip was added. Samples were examined by a pathologist at the University of Liverpool.

2.12 RNA extraction from swabs and allantoic fluid for RT-PCR

For RNA extraction, the phenol chloroform method was used as previously described (Ball *et al.,* 2016c). A volume of 300 µl of TOC media from swabs or AF was treated with 300 µl of solution D (Appendix I), and frozen at -20°C overnight. This was then mixed with 50 µl sodium acetate and 650 µl phenol chloroform 5:1. Tubes were mixed and then centrifuged at 13,000 g for 5 minutes. The top layer was removed and mixed with 500 µl isopropanol and then precipitated overnight at -20°C. Following this, the sample was immediately centrifuged at 13,000 g for 15 minutes. The supernatant was removed and the precipitate was carefully washed twice with 100% ethanol and then left to air dry. The precipitate was re-suspended in 30 µl of re-suspension water (containing 2.5 µl of Rnasin, 5 µl of DTT and 92.5 µl water) and left to stand at 4^{o}C for at least 15 minutes. Samples were then vortexed for 10 seconds before proceeding for RT-PCR (Chomczynski and Sacchi 2006).

2.13 RNA extraction from FTA cards and TOC

FTA cards discs or circles were cut down using sterile scissors and forceps. Then, these discs were placed in tubes contain 1000 ml TE[8] buffer (Chapter 5). After, vortexing and incubating at room temperature for 10 mins 100 µl was taken for extraction. From TOC 140 µl of supernatant was taken and processed for extraction as described below.

Viral RNA was extracted using the QIAamp viral RNA Mini Kit (Qiagen) (Appendix I) according to the manufacturer's instructions. In brief, 140 µl of each sample was added to a 1.5 ml Eppendorf tube[9] containing 560 µl of buffer AVL lysis buffer (containing reconstituted carrier RNA) and vortexed for 15 s. Samples were then incubated at room temperature for 10 minutes and pulse centrifuged. A volume of 560µl of 100% ethanol was added to each tube, vortexed and pulse centrifuged. Then 630 µl was removed from each tube, placed in a spin column/collection tube and centrifuged at 8000 rpm for 1 minute, flow through was discarded and this step was repeated with remaining sample. The spin column was placed in a clean collection tube along with 500 µl of buffer AW1.The previous step was repeated with using AW2 buffer. Spin columns were centrifuged at 13000 rpm for 3 min and the flow through was discarded. Centrifugation was repeated at 13000 for 1 minute. Finally, 60 µl of Sigma water was used to elute the RNA from the column and stored at -20 °C (Awad *et al.*, 2014).

[8] (Thermo Fisher Scientific, USA)

[9] Elkay Laboratory Products UK (Ltd), Basingstoke, Hampshire, UK

2.14 Total RNA extraction from tissues collected on RNA® later

Total RNA extraction was carried out using the RNeasy Mini Plus kit [10]for tissue samples (Appendix I). Samples (30mg) were cut into small pieces using sterile scissors and mixed with 600 µl of RLT buffer plus mercaptoethanol (10µl Mercaptoethanol to each 1 ml RLT buffer) and a stainless steel bead (5 mm). Tissues were homogenised using a Tissue Lyser[11] and lysate was centrifuged at 13000 rpm for 3 min. The solution (550 µl) was transferred to a spin gDNA eliminator spin column. Then, samples were centrifuged for 30 sec at 10,000rpm, flow through was saved in the collection tubes. To a 550 µl of the supernatant similar volume of 550 µl of 70% ethanol was added. From the previous mixture a 700 µl including any precipitate was placed in a RNeasy spin column. Washing and elution was performed according to the manufacturer's instructions using RW1, and RPE buffer in two stages. A volume of 30 µl RNase-free water was used to elute the RNA from the column. A NanoDrop1000 spectrophotometer was used to quantify the extracted RNA. The extracted RNA was stored at -70^{o}C until used for quantitative reverse transcription PCR (qRT-PCR) analysis and preparation of cDNA for host gene and cytokine mRNA expression.

2.15 IBV RT-PCR of the partial S1 gene

The IBV RT-PCR was conducted as previously described (Worthington *et al.,* 2008) that targets a 393bp portion of the S1 gene (Appendix I).

[10] Qiagen

[11] Storm Bullet Blender

2.15.1 Reverse transcriptase

The RT reaction mix comprised of (x5 Buffer, DTT , dNTP's, Rnase inhibitor, Superscript II RT[12], water and SX2- primer) (Table 3.2) (Appendix I). Five µl was added into a 0.2 ml pre labelled Eppendorf tube for each sample, along with 0.5 µl of extracted RNA. The tubes were then placed in a thermocycler[13] and run under the following conditions: 42^{o}C for 1 hour, 72^{o}C for 10 minutes and then held indefinitely at 8 ^{o}C.

2.15.2 Nested PCR 1

A master mix or Supermix containing (DNA polymerase, salts, magnesium, and dNTPs) (Appendix I) was added to the product of reverse transcriptase as follows; 0.5 µl from SX1+ and SX2- respectively (Table 3.2) with 19 ml from Supermix[14]. Samples were run under the following conditions: 94^{o}C for 15 seconds followed by 35 cycles of 94^{o}C for 10 seconds, 50^{o}C for 20 seconds, and 72^{o}C for 40 seconds and then held indefinitely at 8^{o}C.

2.15.3 Nested PCR 2

The master mix was prepared as follows; 24µl from Supermix mixed with 0.5µl- oligo SX3+ and 0.5µl oligo SX4-(Appendix I). After vortexing, a total volume of 24.5µl was placed into 200 µl clip top eppendorf tube (Appendix I).Then, a volume 0.5µl of PCR 1 product was added. Samples were placed in a thermocycler under the following conditions: 94^{o}C for 15 seconds for 1 cycle and 35 cycles at 94^{o}C for 10 seconds, 50^{o}C for 20 seconds and finally 72^{o}C for 40 seconds. Finally, samples were held at 8^{o}C.

[12] Invitrogen

[13] 13GeneAmp PCR system 9700

[14] Thermo Fisher Scientific

Table 2.2. Primers that were used in the partial S1 PCR (PCR1 and PCR2)

Oligonucleotide	Sequence(5' 3')	Position
SX1+a	CACCTAGAGGTTTGTTAGCATG	677-698
SX1+b	CACCTAGAGGTTTGCTTGCATG	
SX2-a	TCCACCTCTATAAACACCTTTAC	1148 -1168
SX2-b	TCCACCTCTATAAACACCCTTAC	
SX3+ a	TAATACTGGCTAATTTTTCAGATGG	705 - 725
SX3+b	TAATACTGGTAATTTTTCAGATGG	
SX4-	AATACAGATTGCTTACAACCACC	1075-1097

*Each following primers SX1, SX2, and SX3 are a combination of two oligonucleotides (a and b).

2.15.4 Agarose gel electrophoresis

A 1.5 % w/v (0.7g in 35 ml of 1x TBE buffer) agarose gel was prepared by dissolving agarose in 1x TBE buffer (Tris-borate-EDTA) by heating in a microwave (Appendix I). After cooling, a 2 µl nucleic acid solution (Redsafe^{TM})[15] was used to stain the agarose. The agarose was then poured into a mould (12cm x 9cm) Hybaid Electro-4 gel tank[16] consist of 20 wells. A gel comb was used to make wells within the gel during the casting period. After 15-20 m set period, the wells in the agarose gel were loaded with 10 µl of product from PCR 2 mixed with 4 µl of loading buffer (Appendix I). A 100 bp ladder (molecular marker)[17] was also included to compare the amplicon size. The gel ran at 75V for 50 min in 1x Tris-borate-EDTA (TBE) buffer (Appendix I), and visualised using an ultra violet

[15]Intron Biotechnology, Inc

[16] Hybaid Ltd, Middlesex, UK

[17] Amersham Pharmacia Biotech, UK

transilluminator[18]. To determine the size of the PCR fragments, amplicons were compared to the DNA ladder.

2.15.5 Purification for sequencing

Purification of the nested PCR product was carried out using 0.99 µl shrimp alkaline phosphatase (SAP)[19] mixed with 0.15 µl Exonuclease 1 (EXO)[20]. The sample was incubated for 30 minutes at 37 °C and then for 10 minutes at 80 °C to remove unwanted substance. Samples were sent for bi-directional sequencing at an external laboratory using forward and reverse primers (Source Bioscience Ltd, Nottingham, UK).

2.16 cDNA synthesis

From the total RNA extraction, the SuperScript III First-strand synthesis system[21] was used to prepare first strand cDNA (Appendix I). The reaction mixture was prepared from random primers, diethyl-pyro carbonate (DEPC) treated water and dNTP mix[22], and 1 µg was added from the RNA sample. The sample was incubated for 5 minutes at 65^{o}C then chilled on ice for 1 minute. Following incubation, a volume of 7 µl of cDNA synthesis mix (First strand buffer, DTT, RNase out inhibitor and Superscript III RT) was added to each primer /RNA mix. Samples were centrifuged at 5,000 rpm for 10 s, followed by incubation at 25^{o}C for 10 min, then 50^{o}C for 50 min, and the reaction was terminated at 85^{o}C for 5 min. At the end of the reaction, tubes were kept on ice.

[18] UVitec Ce Mo32977 transiliminuim UVITEC celt

[19] Qiagen, UK

[20] 78250, Affymetrix

[21] Invitrogen, Life Technologies, UK

[22] Invitrogen, Life Technologies, UK

2.17 Nucleotide comparison of partial S1 gene sequences

Using ChromasPROv1.7.3 the sequences were first cleaned and analysed (http://technelysium.com.au/).Isolate identification was confirmed after a BLAST (National Centre of Biotechnology Information) search was carried out against the full database. Sequences were then aligned in MEGA6 (Tamura *et al.*, 2013), using Clustal W (Thompson *et al.*, 1994). Local BLAST was carried out against the original inoculated viral sequence. A single nucleotide polymorphism (SNP) is a variation of a single nucleotide that occurs at a specific position. All SNPs, insertions and deletions were identified using MEGA6.

2.18 Quantitative reverse transcription PCR (qRT-PCR) for quantification of viral RNA

The IBV 3' untranslated region (UTR) gene-specific primer and probe sequences were used to quantify the viral RNA as previously described (Jones *et al.*, 2011). This protocol uses the IBVRT1 forward primer CTATCGCCAGGGAAATGTC, and the IBVRT2 reverse primer GCGTCCTAGTGCTGTACCC, along with the IBVRT3 TaqMan® probe FAM-CCTGGAAACGAACGGTAGACCCT-TAMRA. The qRT-PCR Roter® gene probe, Real-Time PCR kit[23] (qPCR) (one-step) was utilized according to manufacturer's instructions using 40 ng of total RNA per reaction.

Cycling conditions were 10 min at 50°C and 5 min at 95°C, 40 cycles of 5 s at 95°C, 10 s at 60°C annealing temperature and followed by a final step of 10 s at 72°C. The Rotor-Gene® Q thermocycler software was used to measure the threshold cycle (Ct) after

[23] Qiagen, UK

amplification plots were recorded and analyzed. The relative equivalent units (REU) of viral RNA was identified after the Ct data was converted as previously described (Londt *et al.*, 2013) by standard curve generated via 10-fold dilutions of extracted RNA from infected allantoic fluid of 10^6 EID_{50} of M41.

2.19 Quantitative reverse transcription PCR (qRT-PCR) for host gene expression analysis

The prepared cDNA above was subjected to qRT-PCR to evaluate host gene expression by calculating the relative expression in triplicate. The LightCycler® 480[24] was used to quantify the selected genes with LightCycler 480 SYBR green primers which were supplied by Eurofins Genomics[25] (Table 2.3), and Master Mix 1[26]. Each sample included 10 µl of master mix, 1.8 µl each of forward and reverse primers (each at 300nM) of the selected gene, and 1.4 µl of nuclease free water (Appendix I). The cDNA was diluted at a ratio of 1:50 in a final volume of 5 µl. The diluted cDNA was added to 15 µl of the prepared master mix for a total volume of 20 µl. The cycling conditions were 10 min at 95°C, 45 cycles of 10 s at 95°C, 10 s at 60°C annealing temperature and 10 s at 72°C, followed by a final step of melting curve analysis to ensure the specificity of the SYBR green PCR. Data obtained from the qRT-PCR was normalised by a relative standard curve method to 18S ribosomal RNA (18SrRNA) expression (Kuchipudi *et al.*, 2012; Chhabra *et al.*, 2016), which were presented as fold changes in gene expression of mock vs virus infected samples.

[24] Roche, UK

[25] Edersberg, Germany

[26] Roche, UK

Table 2.3. Primer abbreviations, full names and sequences that used for analyzing pro-inflammatory cytokines and host genes profiles (Kuchipudi *et al.*, 2012).

	Gene**	**Primer sequences: sense (S) and anti-sense (AS)**
Reference gene	18S rRNA*	S) TGTGCCGCTAGAGGTGAAATT
		(AS) TGGCAAATGCTTTCGCTTT
Viral recognition	MDA5	(S) AGGAGGACGACCACGATCTCT
		(AS) CCACCTGTCTGGTCTGCATGT
	LITAF	S) CCCTTCTGAGGCATTTGGAA
		(AS) CAGCCTGCAAATTTTGTCTTCTT
	TLR3	(S) GCAATTTCTCCTTCACCTTTTCA
		(AS) CTTTATGTTTGCTATGTTGTTATTGCT
Type I Interferons	IFN-α	S) CTTCCTCCAAGACAACGATTACAG
		(AS) AGGAACCAGGCACGAGCTT
	IFN-β	S) TCCAACACCTCTTCAACATGCT
		(AS) TGGCGTGTGCGGTCAAT
Inflammation	IL-1β	S) TGCTGGTTTCCATCTCGTATGT
		(AS) CCCAGAGCGGCTATTCCA
	IL-6	(S) CACGATCCGGCAGATGGT
		(AS) TGGGCGGCCGAGTCT

**18S rRNA =ribosomal RNA, MDA5 = Melanoma differentiation-associated protein 5, LITAF=Lipopolysaccharide-induced tumor necrosis factor (TNF)-α factor), TLR3=Toll like receptor 3, IFNα =Interferon alpha, IFNβ =Interferon beta, IL-1β =Interleukin 1 beta, IL-6 =Interleukin 6.

2.20 Enzyme linked immunosorbent assay (ELISA)

Serum samples were analysed using a commercial IBV ELISA kit (IDEXX) according to manufacturer's instructions. Sera were diluted in a ratio of 1:500 with diluent and added to coated antigen plates. Plates were incubated for 30 minutes at 25 ^{o}C then washed 3-5 times with 350 ml of sterile distilled water. Following this, 100 ml conjugate was added then incubated for a further 30 minutes. The wash step was repeated, substrate was added and

further incubated away from direct light. Finally, 100ml of stop solution was added to each well. Plates were read using a micro plate reader Multiskan™ FC Microplate Photometer[27] at 650nm to determine the sample absorbance. Samples with a sample/positive ratio volume greater than 0.2 were considered as positive, the calculation of the titre was achieved by using the formula Log_{10}+1.09(log S/P) +3.36.

2.21 Immunohistochemistry

2.21.1 Cryostat (tissue section)

Approximately 1-2 cm (longitudinal) was taken from each proventriculus and placed in cryo embedding compound optimal cutting temperature (OCT)[28]. The sample was immediately frozen in -190 ^{o}C liquid nitrogen and kept at -80 ^{o}C until required for processing. Sample blocks were sectioned by a pre-cooled cryostat[29] covered with a glass slide and placed in the dark overnight. Fixation was carried out with pre-cooled -20 ^{o}C acetone for 10 minutes. Finally, samples were dried at room temperature (RT) for around 10 minutes and stored at -70^{o}C until staining.

[27] Thermo Fisher Scientific

[28] Solmedia laboratory, Shrewsbury, UK

[29] LEICA CM 1900, Germany

2.21.2 Immunohistochemistry staining (IHC)

Monoclonal antibodies against [30]IBV nucleocapsid protein were used to confirm the presence of IBV antigen in the proventriculuar tissue. Staining was carried out as previously described (Rautenschlein *et al.*, 2011).

Fixed sections of proventriculus were further treated as follows. To prevent solutions running off the slides a ImmEdge Hydrophobic Barrier PAP Pen[31] was used to draw a barrier around the sections and the sections were washed in Tris Buffered Saline (TBS) (Appendix I) for 5 mins. Endogenous peroxidase activity was blocked using 100 µl of Dako REAL[32] for 10 min at room temperature before the primary antibody (monoclonal antibody against N-protein[33]) was applied at a dilution of 1:1000 in TBS. Slides were incubated in the primary antibody overnight in a humidified chamber to prevent the slides drying out. For antibody detection 3x drops of Dako ENVISON+ horseradish peroxidase (HRP) (α mouse) was applied to the slides for 30 min at room temperatures before washing 3x2min in TBS. To produce the insoluble dark brown DAB-HRP substrate DAB (3, 3 - diaminobenzidine) (Impact DAP)[34] was applied to the sections for 3-5 mins (according to manufacturer's instructions) followed by 2x 5min washes in TBS and distilled water respectively. Slides were counterstained in haematoxylin for 1 minute and "blued" in

[30] Southern Biotech, Birmingham, AL, USA

[31] Vector Laboratories

[32] Dako Denmark A/S

[33] prionics, Lelystad, the Netherlands

[34] Impress kit, BURLINGAME, CA, vector lab

running tap water for 5 mins. Finally, slides were dehydrated (1x 96% ethanol 1min, 2x 100% ethanol 3mins) before clearing in xylene and cover slipping with DPX.

2.22 Statistical analysis

All the statistical analysis was done using GraphPad Prism version 6. Data was analysed according to data type (Figure 2.5; obtained from (Lopes *et al.*, 2014). Parametric data was analysed using the one-way analysis of variance (ANOVA), followed by the *post-hoc* least significant difference (LSD) multiple comparison test for more than two groups, and Student's t test if there were two groups. For the trachea and kidney lesion scores and viral load, the significant differences between the groups were analysed by Kruskal-Wallis test followed by Dunn's mean test. Differences between groups were considered significant at $P<0.05$. The Kruskal-Wallis test is considered an acceptable alternative to ANOVA.

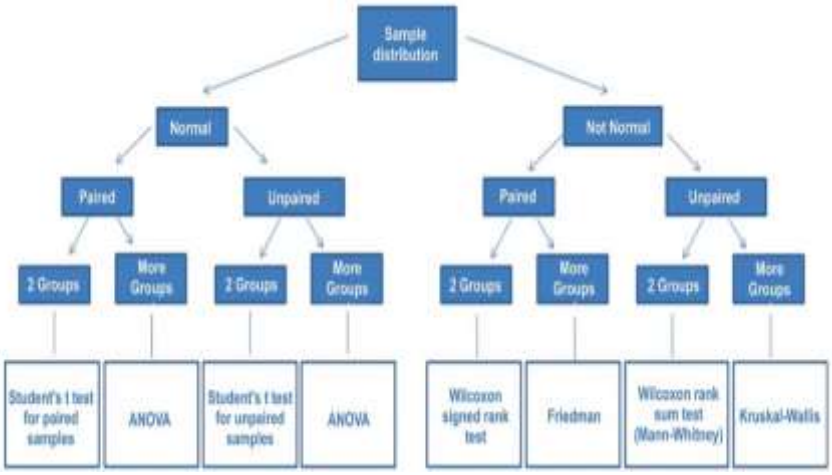

Figure 2.5. Flow chart to indicate the appropriate statistical test according to the data distribution

Chapter 3: Evaluation of the full S1 gene sequencing of Classical and variant infectious bronchitis viruses extracted from allantoic fluid and FTA cards

Abstract

Sequence variability in the S1 gene determines the genotype of infectious bronchitis virus (IBV) strains. An RT-PCR was developed to amplify and sequence the full S1 gene of classical and variant IBVs enriched in allantoic fluid (AF) or the same AF inoculated onto Flinders Technology Association (FTA) cards. Seven IBV strains (M41, D274, 793B, IS/885/00, IS/1494/06, Q1 and QX) were grown in SPF eggs and RNA was extracted from AF. Full S1 gene amplification was achieved by using two primers and products were sequenced using Primers; A, SX3, 1050+ and 1380+ to achieve full S1 gene coverage. Following serial dilutions of AF, it was found that detection limits of the partial assay were higher than those of the full S1 gene. Partial S1 sequences exhibited higher than average nucleotide similarity percentages (79%; 352bp) compared to full S1 sequences (77%; 1,756bp), suggesting that the full S1 protocol has greater strain differentiation accuracy. For IBV detection from AF inoculated FTA cards, four serotypes were incubated up to 21 days at three temperatures; 4^{o}C, room temperature (24^{o}C) and 40^{o}C. RNA was extracted and tested with partial and full S1 protocols. Through partial sequencing, all IBVs were successfully detected at all sampling points and storage temperatures. In contrast, for full S1 sequencing, was not able to amplify the gene beyond 14 days of storage or when stored at 40^{o}C. Full S1 sequencing appears to be suited for detection of IBVs enriched in AF, and has limited application for samples directly embedded onto FTA cards.

3.1 Introduction

Infectious bronchitis (IB) is an acute viral disease caused by infectious bronchitis viruses (IBVs), that mainly infect the respiratory system in broiler and layer chicken flocks (Cavanagh 2005). Infection causes economic losses due to high mortality, poor body weight gain (Ignjatovic and Sapats 2000), a decrease in egg production (reaching up to 70%) and reduction in egg quality (increased eggshell pigment) in layers (Box and Ellis 1985; Muneer *et al.,* 2000). In some cases, infection can cause further complications, including cystic oviducts (Benyeda *et al.,* 2009; Ganapathy *et al.,* 2012) and false layer syndrome (Crinion and Hofstad 1972a). Certain IBVs, including several Australian strains, have been associated with renal complications such as nephritis (Cavanagh 2005; Mahmood *et al.,* 2011) and digestive complications, such as proventriculitis (Ganapathy *et al.,* 2012; Toffan *et al.,* 2013).

IBV is a single stranded RNA virus (approximately 27.6kb in size) belonging to the family *Coronaviradae*. As with other coronaviruses, IBV contains four structural proteins: spike protein (S), small membrane envelope protein (E), membrane protein (M), and nucleoprotein (N). The spike protein comprises 1,145 amino acids, with functions including attachment to host cells, neutralization of antibodies and initiation of protective immunity (Ignjatovic and Galli 1994; Johnson *et al.,* 2003). It undergoes post translational cleavage to form S1 and S2 subunits (Cavanagh *et al.,* 1986; Jackwood and de Wit 2013). Furthermore, it is the most variable of the IBV proteins, with most mutations and recombination events mapping to the S1 region (Adzhar *et al.,* 1997; Kingham *et al.,* 2000), which can lead to the emergence of new variant strains (Zhang *et al.,* 2015).

The relationship between antigenic differences among IBV strains has been previously investigated (Raj and Jones 1997b; Jackwood and de Wit 2013), and others have reported

that partial S1 sequences have a stronger correlation with protective relatedness between strains, rather than antigenic relatedness (Ladman *et al.,* 2006). In addition, variations in the nucleotide sequence of S1 may lead to alterations in the virus categorisation, such as receptor binding abilities(Jackwood and de Wit 2013).

More than 25 serotypes have been recognized to date, often differing by up to 50% at the amino acid level (Adzhar *et al.,* 1997; Gelb Jr *et al.,* 2005). Only a minor amino acid sequence alteration, at the very least a 5% change (25-30 amino acids) in the S1 subunit, is required to affect the protection status conferred by vaccinations of the same serotype (Cavanagh 2003). As a result, there is low cross protection among vaccine serotypes (Kuo *et al.,* 2010).

In recent years, sequence analysis has become a common method to identify strain types, typically being carried out on specific nucleotide regions of the S gene (Cavanagh 2003) or the N gene (Williams *et al.,* 1992). Previous studies have focused on analysing different base pair portions of the S1 gene (between 228 to 380bp) (Wang and Tsai 1996; Al-Shekaili *et al.,* 2014), leading to difficulties when comparing genotype analysis between studies.

For many years, the Massachusetts (Mass) serotype was the only recognized serotype (via virus neutralisation), originally isolated in Massachusetts in the 1930s. Jungherr and colleagues reported a Connecticut IBV type in 1956, which was not cross protective to nor cross neutralised by the Mass IBV strain (Jungherr *et al.,* 1956). In 1984, researchers in the Doorn institute first isolated the Dutch strain D207 from Mass vaccinated flocks (Davelaar *et al.,* 1984). The 793B serotype was identified in the 1990s (Parsons et al., 1992), with sequence data distinct from Dutch and Mass (Cavanagh 2005). The QX genotype was first described in 1996 in China, and was associated with severe nephritis, proventriculitis,

tracheitis, decreased egg production and false layer syndrome (RC Jones 2010; JJ De Wit *et al.,* 2011). Later, another variant, known as Q1, was detected from layer flocks showing respiratory signs, which can also cause proventriculitis(Yu *et al.,* 2001). While certain genotypes like 793B and Mass have a global distribution (Cook *et al.,* 1996), others are considered to have regional importance, such as IS/885/00 (Meir *et al.,* 2004), IS/1494/06 (GenBank accesion number EU780077), Arkansas (Ark) (JJ De Wit *et al.,* 2011) and the California variants (Moore *et al.,* 1998).

Recently, there has been growing interest in complete S1 sequencing, with reports from Thailand, Sweden and India (amongst others) aiming to further understand the molecular characterisation of IBV (Pohuang *et al.,* 2011; Abro *et al.,* 2012; Kamble *et al.,* 2014). In addition, it was suggested, during the 8th international symposium on avian corona-pneumoviruses and complicating pathogens (2014), that the full S1 gene should be sequenced to determine the genotype of IBV isolates. Recent work in Italy, has outlined the benefits of sequencing the full S1 gene and classified IBV strains into a number of distinct lineages based on the data (Valastro *et al.,* 2016). An earlier study aimed to obtain full S1 sequences by using two primers to amplify and sequence around 1,700 base pairs (bp) from the S1 gene, after purifying and digesting with restriction enzymes (Kwon *et al.,* 1993). Recently, the same primers were used in a second study (Abdel-Moneim *et al.,* 2006), while a separate investigation looked into the differences in sequencing the entire S1 and partial N gene for grouping IBV genotypes (Huang *et al.,* 2004).

Flinders Technology Association (FTA) cards have been used for a number of IBV studies looking to detect the virus from clinical or laboratory samples (Moscoso *et al.,* 2005; Ganapathy *et al.,* 2015). While the cards are useful for transporting genomic material worldwide, the quality and quantity of RNA eluted varies depending on the initial amount

of IBV RNA, storage duration and temperature and the extraction methodology. Previous work has outlined that for IBV, a partial S1 amplification and sequencing from FTA card extraction is successful down to a viral concentration of $10^{0.2}$ EID_{50}/ml (Awad *et al.*, 2012). However, the feasibility of full S1 gene sequencing has not been examined. For IBVs extracted from FTA cards, it has been reported that storage duration and temperature can influence the molecular detection of several IBV strains(Moscoso *et al.*, 2005).

The aim of this study was to develop and evaluate a RT-PCR assay to amplify and sequence the full S1 gene of seven IBV genotypes grown in allantoic fluid. The sensitivity of the scheme was investigated using RNA extracted from allantoic fluid, both directly and after inoculation onto Flinders Technology Association (FTA) cards. The effects of storage temperatures on detectability of the viruses were also assessed.

3.2 Materials and methods

3.2.1 Virus strains

The following virulent IBV strains were used in this study: M41, QX, D274, IS/1494/06, IS/885/00, Q1 and 793B (Ganapathy *et al.*, 2012; Chhabra *et al.*, 2015b). Viruses were passaged in embryonated specific-pathogen free (SPF) chicken eggs (Chapter 2.1).

3.2.2 Strain titration in tracheal organ cultures (TOCs):

All the strains used in this study were passaged in embryonated specific-pathogen free (SPF) chicken eggs and titrated in tracheal organ cultures (TOCs) to obtain the ciliostatic dose per ml (CD50/ml) (CD50) (Chapter 2.2) (Cook *et al.*, 1976b). The initial titres for the viruses are given in the bracket; 793B ($10^{6.4}$ CD_{50}/ml), M41 ($10^{6.3}$ CD_{50}/ml), D274 ($10^{6.75}$ CD_{50}/ml), QX ($10^{6.3}$ CD_{50}/ml), Q1 ($10^{5.5}$ CD_{50}/ml), IS/1494/06 ($10^{6.3}$ CD_{50}/ml) and IS/885/00 ($10^{6.3}$ CD_{50}/ml)

3.2.3 Inoculation of FTA cards:

FTA cards[35] were inoculated with 100µl (Figure 3.1A) of ten-fold diluted allantoic fluid in TOC media (Chapter 2.2) for each virulent strain after carefully handling and labelling. The inoculated FTA cards were stored at three different temperatures; 4°C, 40°C (Figure 3.1B) and room temperature (RT, 22-26 °C). For each sampling point (1, 2, 3, 7, 14, 21 days post inoculation), the extraction was carried out from one disc (FTA cards).

3.2.3 RNA extraction

3.2.3.1 Allantoic fluid

In our laboratory, used laboratory strains in this study of the afore mentioned viruses were growing and passaged grown in embryonated eggs, the allantoic fluid was collected and stored until used for either titration in TOCs or FTA cards inoculation. From the AF, the viral RNA was extracted using the QIAamp viral RNA Mini Kit[36], according to the manufacturer's instructions, from 100 µl of neat and tenfold diluted allantoic fluid (in TOC media). In brief, seven labelled tubes (1-7) were. A 10 fold dilution of 100 µl of the warm (37 ^{o}C) AF was added to a 900 µl of warm TOC media (after vortex and changing the pipettes tips) a 100 µl volume was removed and the process was repeated until the dilution 10^{7} was reached (Tube number 7). The AF also used directly for extraction undiluted (3.11).

[35] (WhatmanTM)

[36] (Qiagen, UK)

3.2.3.2 Extraction from FTA cards

One complete disc from the FTA card was removed (of each dilution) using sterile scissors, placed into 1000 µl of TE buffer[37] [10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)] and incubated for 10-15 minutes for elution (Sakai *et al.*, 2015). After vortexing, 100 µl was taken and processed for RNA extraction using the QIAamp viral RNA Mini Kit [38]according to manufacturing instructions (Chapter 2.13).

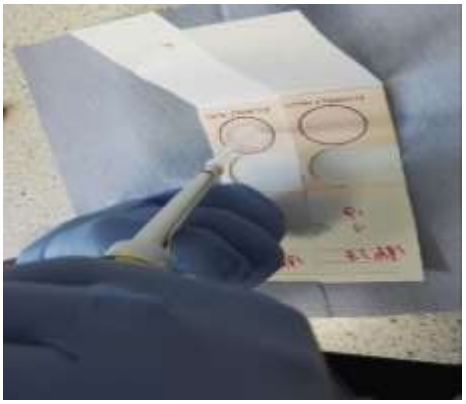

Figure 3.1. Inoculation and storage of FTA cards, inoculation a 100 µl of FTA cards from either neat AF or diluted (tenfold), and left to air dry for 1 hour, and then moved to different storage conditions (4^{o}C, 40^{o}C and (RT).

3.2.4 IBV RT-PCR

Partial S1. Extracted RNA was used for both partial and full S1 amplifications. For partial S1 amplification, RT-PCR targeting a 393bp partial sequence of the S1 gene was

[37] (Thermo Fisher Scientific, USA)

[38] (Qiagen, UK)

conducted, as previously described (Ganapathy *et al.*, 2015; Ball *et al.*, 2016c) followed by gel electrophoresis (Chapter 2.15).

Full S1. During the process for identifying the correct primers, the different pairs tested are presented in (Table 3.1) in order to obtain the full S1 amplification and sequences. However, for successful full S1 amplification, reverse transcription was conducted using the primer 22.51 which is located at the 22.51 KB position (Figure 3.2). The reaction mix comprised 5x first strand buffer [250 mM Tris-HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$[39]], dithiothreitol (DTT) [100 mM[40]], deoxynucleotidetriphosphates (dNTPs)(deoxyadenosine triphosphate (dATP), deoxyguanosine triphosphate (dGTP), deoxycytidine triphosphate (dCTP), deoxythymidine triphosphate (dTTP)) [10mM], RNasin ribonuclease inhibitor [20 U/µl;41], Superscript III Reverse Transcriptase [200U/µl;42], oligonucleotide [10 pmol (picomoles per litre)], sterile distilled water [2.13 µl] and 0.5 µl of template RNA. RT-PCR conditions were: 48°C for 45 min, and 94°C for 5 min. Generated complementary DNA (cDNA) was then amplified in a one-step PCR using primer A (Figure 4.2) and primer 22.51 (Table 4.2). The PCR mixture included PCR Supermix[43] [22 mM Tris-HCl (pH 8.4), 55 mM KCl, 1.65 mM $MgCl_2$, 220 µM dNTPs, 22

[39] Invitrogen

[40] Invitrogen

[41] Promega

[42] Invitrogen

[43] Invitrogen

U/ml recombinant Taq DNA polymerase, and stabilizers] for efficient PCR amplification, forward and reverse primers [10 pmol] and 0.5 µl of cDNA.

Cycling conditions were: 35 cycles of denaturation at 94 °C for 60 s, annealing at 54 °C for 30 s, extension at 72°C for 60 s, and a final extension at 72°C for 10 min. These conditions were found to be the optimal after the process of optimization after different attempts. For example, different voltage were tried and different time for agarose and with super script II and different ions (Mg+2) concentrations. The same for the RT stage and PCR amplifications when different primes and or different times were tested.

Table 3.1. Primers tested at the process of optimization for amplification and sequencing of IBV

Oligonucleotide	Sequence 5'3'	Length	Reference
Primer 22.06 -	CATATCTTCTTTTTGACC	18	In-house
Primer 50 (F)	AAACTGAACAAAAGAGACACTTAG	24	(Zulperi *et al.,* 2009)
IBVc2 (R)	GCCATAACTAACATATGG ACAA	22	(Zulperi *et al.,* 2009)
Primer 745 +	YGAYRRNTCMCCHAVRGGYT	20	In-house
Primer 1.57 -	YTGYTGGTTRACATCTTCRC	21	In-house
Primer 1.45+	TYTRGCWGAYGSHGGKTTRG	20	In-house
Primer 1.77-	TGGCAAATTGAGCCATCAGGT	21	In-house
RT QX S1 neg	CATCTTTAACGAACCATCTGG	21	M. Falchieri *et al.*, 2013
QX S1 start+	CCAGTTGTGAATTTGAAGAAAGAACAA-AAGACCGACTTAG	40	M. Falchieri *et al.*, 2013
QX S1 end	CGAACCATCTGGTTCAATACAAAATCTGC	29	M. Falchieri *et al.*, 2013

Table 3.2. Primers used for successful amplification and sequencing of all IBV strains used in this study.

Oligonucleotide	Sequence 5'3'	Length	Reference
Primer A	GCCAGTTGTTAATTTGAAAAC	19	(Pohuang *et al.,* 2011)
Primer 22.51	GAACGTCTAAAACGACGTGTTCC	23	In house
1050+	GGTTTAATTCCTTGTCAGTTTCTCTTACTTATGG	34	(M. Falchieri *et al*., 2013)
1380+	GCTGCTAATTTTAGTTATTTAGCAGATGGTGG	32	(M. Falchieri *et al*., 2013)
SX3+	TAATACTGG C/T AATTTTTCAGA	21	(Worthington *et al*., 2008)

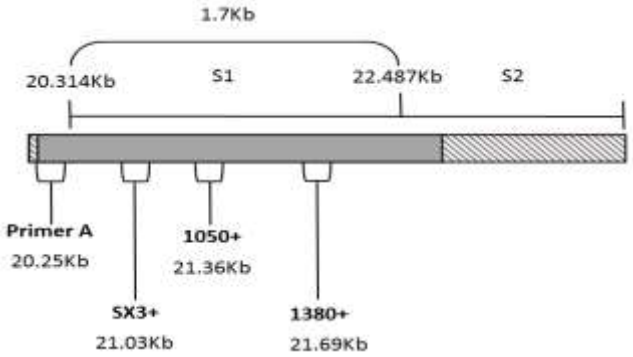

Figure 3.2. Schematic representation of the four primer binding sites (Kb= kilo base) used to sequence the full S1 gene. The grey bar indicates sequence coverage by the scheme.

3.2.5 Agar gel electrophoreses

The results of RT-PCR reactions were verified using agarose gel electrophoresis. After either partial or full S1 PCR, the PCR products were examined by electrophoresis on a 1.5 % agarose gel (Chapter 2.15.4) with the exception of full S1 electrophoresis when the duration was changed to 75 minutes as compared to 50 minutes for the partial S1 sequence.

3.2.6 Cleaning of the positive PCR products

For full S1 products (more than 1700 bp, and partial S1 around 400bp (Figure 4.3)) were visualised on an agarose gel, and positive amplicons were purified[44] (as mentioned in details below and sent for commercial sequencing[45] http://www.ncbi.nlm.nih.gov/)) using the following primers; primer A (Pohuang *et al.*, 2011), SX3+(Worthington *et al.*, 2008) 1050+ and 1380+ (Falchieri *et al.*, 2013) (Figure 3.2). As our amplification primers were not able to amplify the QX strain, a previously published RT-PCR scheme was utilised (Falchieri *et al.*, 2013). In brief, the primer RT QX negative 5'-CATCTTTAACGAACCATCTGG-3' was used in the RT-step. Further two primers QX S1 start positive 5'-CCAGTTGTGAATTTGAAGAAAGAACAAAAGACCGACTTAG-3' and QX S1 end negative 5'-CGAACCATCTGGTTCAATACAAAATCTGC-3' were used in one step PCR amplification.

[44] ExoSAP-IT, Affymetrix, Santa Clara, CA)

[45] (Source BioScience

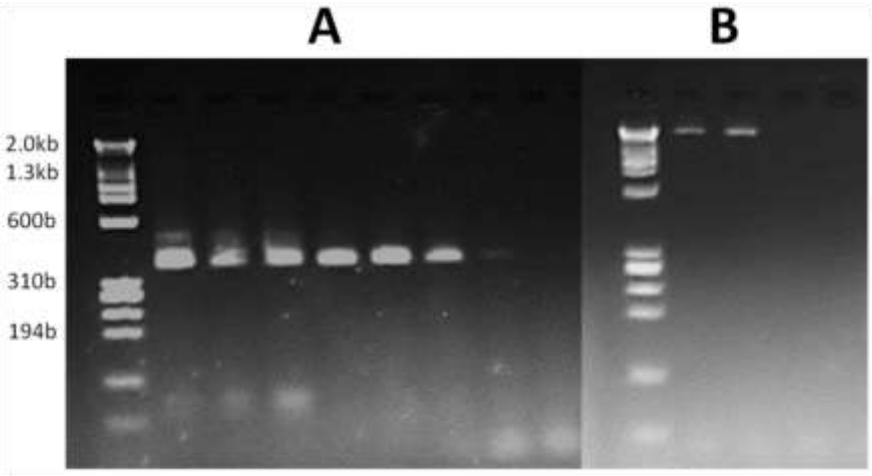

Figure 3.3. Detection of IBV genotypes strains used in the current study using the partial or full S1 schemes. (A) Sensitivity testing of partial S1 RT-PCR assay applied to 793B infected allantoic fluid. DNA marker (lane 1), serial dilutions of 793B infected allantoic fluid (lanes 2-9). (B) Sensitivity testing of full S1 RT-PCR assay applied to 793B infected allantoic fluid. DNA marker (lane 1), serial dilutions of 793B infected allantoic fluid (lanes 2-5).

3.2.7 Purification for sequencing

All the Purification process of the nested PCR product (Chapter 2.15.5). Using shrimp alkaline phosphatase (SAP) mixed with Exonuclease 1 (EXO).

3.2.8 Total quantification of the RNA by Nanodrop

The NanoDrop™[46] 1000 Spectrophotometer from total volume of 1 µl samples with remarkable reproducibility was used to quantify the total RNA extracted from FTA cards at different storage conditions and using R programme to present the readings.

3.2.9 Elution of RNA from FTA cards

After cutting the FTA cards aseptically, and were put in tubes containing TE buffer. We have tried to increase the elution time, this step was carried out in order to increase the yield of RNA from FTA cards by increasing the time up to 24 h with vortexing and shaking.

3.2.9 IBV qRT-PCR

Quantification of the viral RNA was carried out using qRT-PCR as previously described (Jones *et al.*, 2011). All reactions were performed using the One-Step RT-PCR Qiagen kit and 40 ng of total RNA per reaction (Chapter 2.18).

3.2.10 Nucleotide and amino acid comparison of partial and full S1 and gene sequences

Using ChromasPROv1.7.3 the sequences were first cleaned and analysed (http://technelysium.com.au/), then, the isolate identification was confirmed after BLAST search (National Centre of Biotechnology Information) was carried out to the nucleotide alignment in Molecular Evolutionary Genetics Analysis (MEGA6) (Tamura *et al.*, 2013), using Clustal W, a multiple sequence alignment programs (Thompson *et al.*, 1994).

[46] Thermofscientific

BLAST, which is an algorithm for comparing primary biological sequence information, such as the nucleotides of DNA sequences or amino-acid sequences of proteins. BLAST analyses were carried out against the original inoculated neat viral sequence. Nucleotide and amino acid variations were carried out using MEGA6.

3.2.11 Phylogenetic analysis

Reads for the full S1 sequencing were initially examined for their quality and assembled using ChromasPRO v1.7.3[47]. Sequences were then trimmed to cover the full S1 gene. Partial S1 sequences (350bp) were processed using the same methodology. BLAST comparisons against the NCBI database (GeneBank) were carried out to confirm strain identity. Alignments for all S1 sequences, and reference genomes, were carried out in MEGA6 (Tamura *et al.*, 2013) maximum likelihood method with 1000 bootstrap replicates. Reference strains used were D274 (X15832), 793B (AF093794), IS/885/00 (AY279533), IS/1494/06 (EU780077), Q1 (AF286302), QX (KF297571), M41 (AY561711), H120 vaccine C (KU736750), H120 vaccine D (KU736751), Ma5 vaccine (KU736747), Beaudette strain (DQ001336), Ark-DPI (KX529820), IBV Quebec strain (AF349621), IBV T strain (AY775779) and Connecticut vaccine (EU283058).

3.2.12 Field samples

Twenty-four tissues and swab samples were collected from flocks and imprinted on FTA cards in the Middle East and were previously confirmed to carry an IBV infection. All

[47] http//technelysium.com.au/)

RNA was subjected to our full S1 RT-PCR as previously described in this study (Ganapathy *et al.*, 2015). The details of samples are illustrated in (Table 3.3).

3.2.13 Analysing of RNA yield readings

To this the R programme for statistician was used to present the data on the days post inoculation and according to the dilutions that were used for inoculation FTA cards then storage them on the mentioned different temperatures.

Table 3.3. The sample identity of tissues or swabs that were used for detection of IBV by partial S1 RT-PCR and sequencing. Tr= trachea, K= kidney, OP= oropharyngeal, CL= cloacal swabs, L=Liver, CT=Cecal tonsils

	Laboratory ID	**Date Received**	**IBV genotype (Partial S1)**	**Sample type**	**Positive full S1**
1	KG9/17.2	20/03/2017	793B	FTA Card/Spleen	
2	KG2/17.1	24/01/2017	793B	Swab/K	
3	KG53/16.2	19/10/1016	793B	Swab/OP	
4	KG16/16.5	07/04/2016	793B	Swab/CL	
5	KG10/16.1	22/03/2016	793B	Swab/CL	
6	KG29/15.27	30/06/2015	793B	FTA card/CT	
7	KG18/15.10	01/05/2015	793B	FTA card/Tr	
8	KG12/15.3	07/04/2015	793B	FTA card	
9	KG4/15.27	27/01/2015	793B	FTA card/CT	Yes
10	KG57/16.1	21/11/2016	Mass	FTA Card/AF	
11	KG12/16.1	24/03/2016	Mass	Swab/Tr	
12	KG11/16.1	23/03/2016	Mass	Swab/Tr	
13	KG29/15.9	30/06/2015	Mass	FTA card/Tr	
14	KG23/15.9	11/06/2015	Mass	FTA card/Tr	
15	KG18/15.35	01/05/2015	Mass	FTA card/CT	
16	KG14/15.2	09/04/2015	Mass	FTA card/L	
17	KG7/15.8	06/02/2015	Mass	FTA card/K	
18	KG46/14.15	22/10/2014	IS/1494/06	FTA card/Tr	
29	KG46/14.31	22/10/2014	IS/1494/07	FTA card/K	Yes
20	KG46/14.29	22/10/2014	IS/1494/08	FTA card/K	
21	KG35/14.21	29/08/2014	IS/1494/09	FTA card/Tr	
22	KG35/14.22	29/08/2014	IS/1494/10	FTA card/Tr	
23	KG7/15.4	06/02/2015	Q1	FTA card/K	
24	KG7/15.5	06/02/2015	IS/885/00	FTA card/Tr	

3.3 Results

4.3.1 Detection limits for partial versus full S1 from allantoic fluid

Results showed that the minimum detection limits for the full S1 gene were 10^{-2} for all strains except for M41, which was 10^{-3}. In contrast, the detection limits for the partial S1 detection were at 10^{-7} for all strains except for Q1, which was 10^{-6} (Table 3. 4).

3.3.2 qRT-PCR from allantoic fluid

The virus enriched allantoic fluid was analysed by PCR to measure the number of viral copies, and 793B, M41 and QX viruses were detected until dilution factor 10^5. While, the other viruses were detected until dilution factor 10^4 (Table 3.4).

Table 3.4. The initial virus titre and the partial and full S1 detection limits from diluted allantoic fluid.

IBV Strain	Titre (CD_{50}/ml)	Detection limits		
		Allantoic fluid		
		Partial S1	*Full S1*	*Real time q PCR detection limits*
793B	$10^{6.38}$	10^{-7}	10^{-2}	10^{-5}
M41	$10^{6.33}$	10^{-7}	10^{-3}	10^{-5}
D274	$10^{6.75}$	10^{-7}	10^{-2}	10^{-4}
QX	$10^{6.39}$	10^{-7}	10^{-2}	10^{-5}
Q1	$10^{5.5}$	10^{-6}	10^{-2}	10^{-4}
IS/885/00	$10^{6.5}$	10^{-7}	10^{-2}	10^{-4}
IS/1494/06	$10^{6.5}$	10^{-7}	10^{-2}	10^{-4}

3.3.3 Nucleotide sequence and phylogenetic analysis

The seven partial S1 sequences exhibited a higher than average nucleotide homology percentage to each other (79%) when compared to the full S1 sequences (77%) (Table 3.5). This was true for both the classical and variant strains. M41 demonstrated a homology of

77% (full) and 80% (partial) to D274, whereas the closely related IS/885/00 strain had 82% (full) and 83% (partial) homology with IS/1494/06. Similarly, the Q1 strain had 77% (full) and 79% (partial) homology with QX.

Phylogenetic analysis further confirmed that the full S1 sequences were more distantly related between serotypes (Figure 3.4) compared to the partial S1 sequences (Figure 3.5) and this was clear when both sequences were combined in the phylogenetic analysis (Figure 3.6).

Table 3.5. Nucleotide identity (%) comparison of the seven IBV strains used in this study based on the full S1 and partial S1.

Nucleotide identity (%) / Full S1								
	M41	*D274*	*793B*	*IS/885/00*	*IS/1494/06*	*Q1*	*QX*	
M41		77	73	77	79	73	72	*M41*
D274	80		77	82	84	79	77	*D274*
793B	80	81		79	77	75	76	*793B*
IS/885/00	84	79	82		82	79	73	*IS/885/00*
IS/1494/06	81	79	79	83		82	81	*IS/1494/06*
Q1	82	80	81	83	80		77	*Q1*
QX	77	75	78	72	74	79		*QX*
	M41	*D274*	*793B*	*IS/885/00*	*IS/1494/06*	*Q1*	*QX*	
Nucleotide identity (%) / Partial S1								

3.3.4 Amino acid analysis

Similar to the nucleotide sequences, full S1 amino acid sequences had a lower homology compared to partial sequences overall (Table 3.6). Strain comparisons showed that M41 had 77% and 73% similarity to D274 and 793B respectively, compared to the partial amino acid sequence which was higher (80% for both). IS/885/00 showed 82% (full) and 83% (partial) compared with IS/1494/06. Similarly, Q1 had 77% (full) and 79% (partial) compared with QX.

Table 3.6. Amino acid identity (%) comparison of the seven IBV strains used in this study based on the full S1 and partial S1.

Amino acid identity (%) / Full S1								
	M41	*D274*	*793B*	*IS/885/00*	*IS/1494/06*	*Q1*	*QX*	
M41		62	53	60	52	56	52	*M41*
D274	76		61	66	73	63	60	*D274*
793B	72	74		65	62	57	58	*793B*
IS/885/00	77	78	78		67	63	54	*IS/885/00*
IS/1494/06	74	79	75	85		55	61	*IS/1494/06*
Q1	76	83	77	79	82		56	*Q1*
QX	53	56	59	48	52	75		*QX*
	M41	*D274*	*793B*	*IS/885/00*	*IS/1494/06*	*Q1*	*QX*	
Amino acid identity (%) / Partial S1								

.

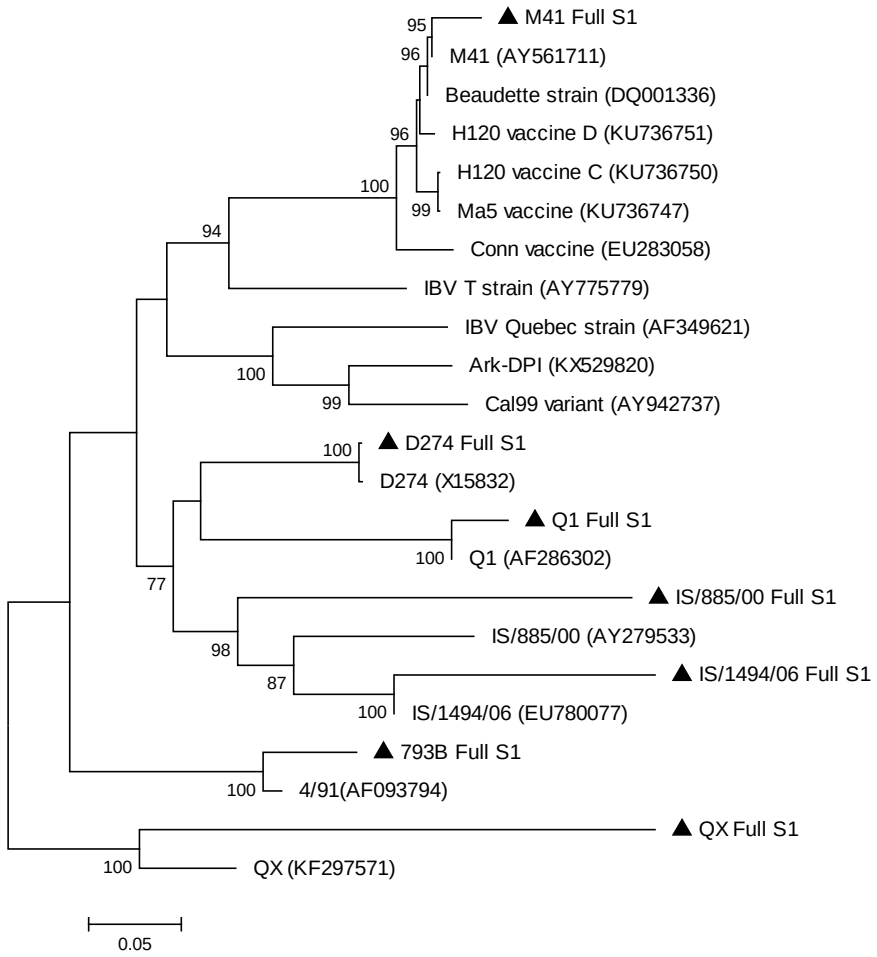

Figure 3. 4. Phylogenetic tree based on full S1 nucleotide sequence among the IBV strains used in this study (filled triangle) and reference strains (between brackets). Analysis inferred using maximum likelihood analysis with Tamura 3-parameter and 1000 bootstrap replicates.

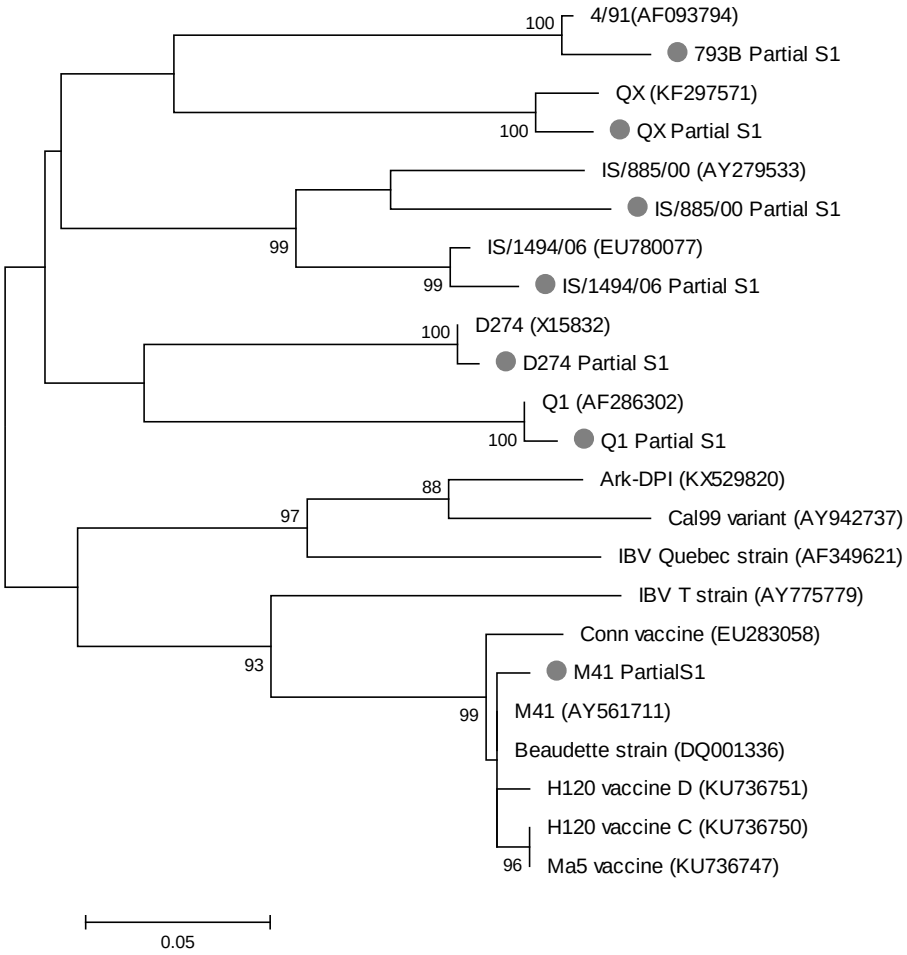

Figure 3.5. Phylogenetic tree based on partial S1 nucleotide sequence among the IBV strains used in this study (filled circle) and reference strains (between brackets). Analysis inferred using maximum likelihood analysis with Tamura 3-parameter and 1000 bootstrap replicates.

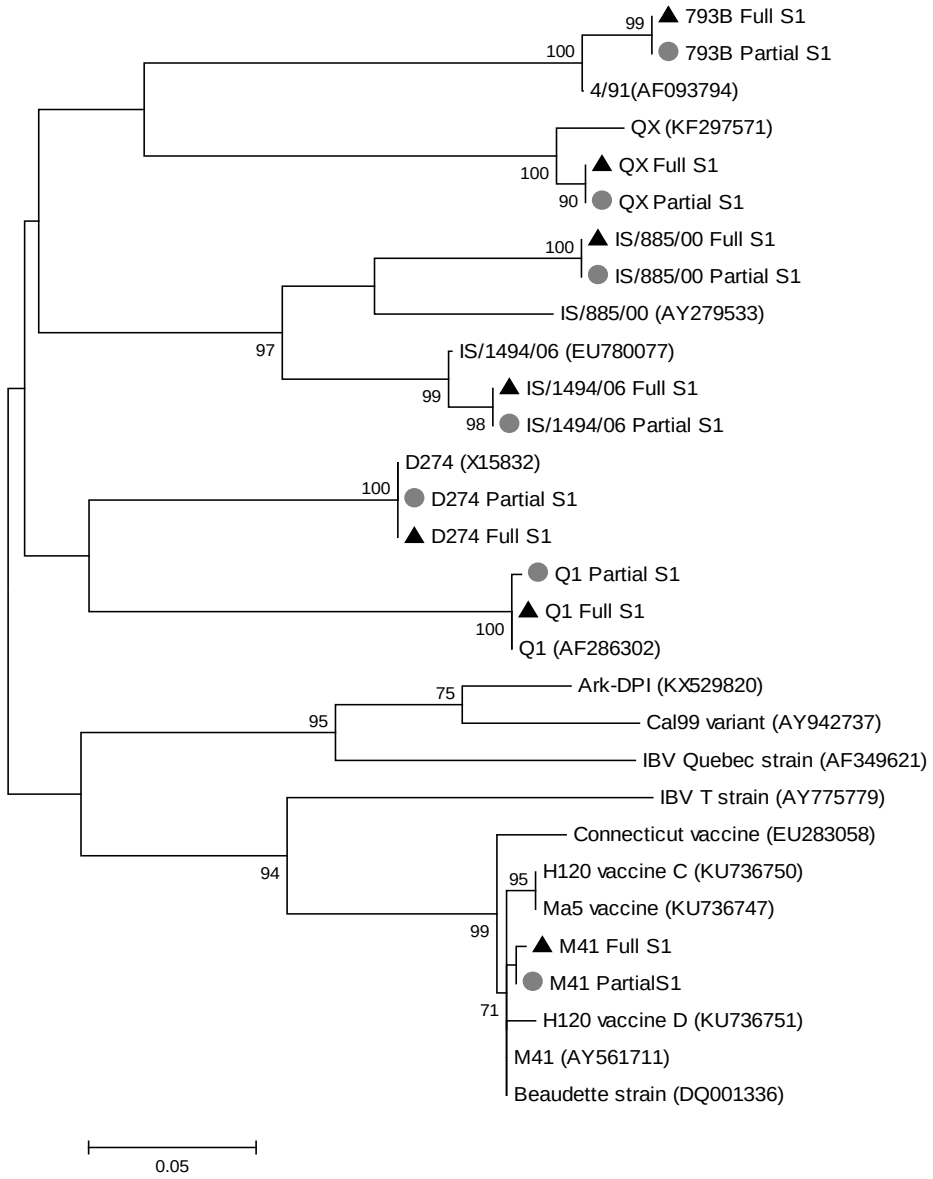

Figure 3.6. Phylogenetic tree based on both the partial (filled circle) and full S1 (filled triangle) nucleotide sequence among the IBV strains used in this study and reference strains (filled triangles). Analysis inferred using maximum likelihood analysis with Tamura 3-parameter and 1000 bootstrap replicates.

3.3.5 Sensitivity of IBV detection following inoculation onto FTA cards

IBV detection using both full and partial S1 RT-PCR varied between strains. The partial RT-PCR detected all strains, at all temperatures, up to 21 dpi. However, the assay was least sensitive for the FTA cards stored at 40^{o}C at 21 dpi. For example, IS/1494/06 was only detected at the neat concentration for 40^{o}C, compared to RT (10^{-3}) and 4^{o}C (10^{-4}) (Table 3.7).

For the full S1 detection, IBV was detected only from the cards stored at 4^{o}C and RT (Table 3.8). Additionally, the lowest dilution we identified IBV at was 10^{-1}. No cards stored at 40^{o}C were positive for the full S1 gene, and no IBV was detected at any temperatures past 14 dpi.

Table 3.7. IBV detection using the partial-S1 assay from FTA cards following IBV strain inoculation and up to 21 days incubation at varying temperatures

	Strain											
	M41			***793B***			***IS/1494/06***			***Q1***		
	Temperature (^{o}C)											
DPI	*4*	*40*	*24*	*4*	*40*	*24*	*4*	*40*	*24*	*4*	*40*	*24*
1	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-4}
2	10^{-3}	10^{-2}	10^{-3}	10^{-4}	10^{-3}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-4}
3	10^{-5}	10^{-3}	10^{-4}	10^{-4}	10^{-3}	10^{-4}	10^{-2}	10^{-4}	10^{-4}	10^{-4}	10^{-3}	10^{-4}
7	10^{-4}	10^{-1}	10^{-3}	10^{-3}	10^{-2}	10^{-3}	10^{-3}	10^{-2}	10^{-2}	10^{-3}	10^{-3}	10^{-3}
14	10^{-5}	10^{-3}	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-4}	10^{-1}	10^{-5}	10^{-3}	10^{-3}	10^{-4}
21	10^{-5}	10^{-3}	10^{-4}	10^{-4}	10^{-3}	10^{-3}	10^{-4}	+*	10^{-3}	10^{-5}	10^{-1}	10^{-3}

Table 3.8. IBV detection using the full S1 assay from FTA cards following IBV strain inoculation and up to 21 days incubation at varying temperatures.

	Strain											
	M41			***793B***			***IS/1494/06***			***Q1***		
	Temperature (^{o}C)											
DPI	*4*	*40*	*24*	*4*	*40*	*24*	*4*	*40*	*24*	*4*	*40*	*24*
1	10^{-1}	-	10^{-1}	10^{-1}	-	10^{-1}	10^{-1}	-	10^{-1}	10^{-1}	-	10^{-1}
2	10^{-1}	-	+	10^{-1}	-	10^{-1}	10^{-1}	-	10^{-1}	10^{-1}	-	+
3	10^{-1}	-	+	10^{-1}	-	-	10^{-1}	-	10^{-1}	10^{-1}	-	-
7	+	-	-	+	-	-	+	-	-	10^{-1}	-	-
14	+	-	-	-	-	-	-	-	-	-	-	-
21	-	-	-	-	-	-	-	-	-	-	-	-

(+) Only the neat concentration was detected (-) No IBV was detected.

3.3.6 Total RNA from extracted FTA cards

We have subjected RNA extracted from FTA cards to the Nano drop for quantification of the nucleic acid present. Approximately all the strains' RNAs concentration dropped down at 7 dpi to around 40nm from a starting range of 270 from (1 mg/microliter) irrespective of the genotypes or the stored temperature, all the RNAs started to degrade after seven days post inoculation.

3.3.7 Effect of elution time on RNA yield

The extraction of RNA from FTA cards was attempted after using longer time up to 24h with vortexing and shaking procedures. Results revealed no changes in the RNA yield and no changes in the sensitivity of full S1 scheme from FTA cards extracted RNA.

3.3.8 Sensitivity of IBV detection from field samples

Twenty-five samples from FTA cards were tested using the full S1 approach (76% from tissue samples and 24% swabs), of which all were positive by partial S1 protocol. Only

three positive samples out of the submitted 25 were positive by full S1 of which sequencing was possible on two samples (not included in Figure 3.7).

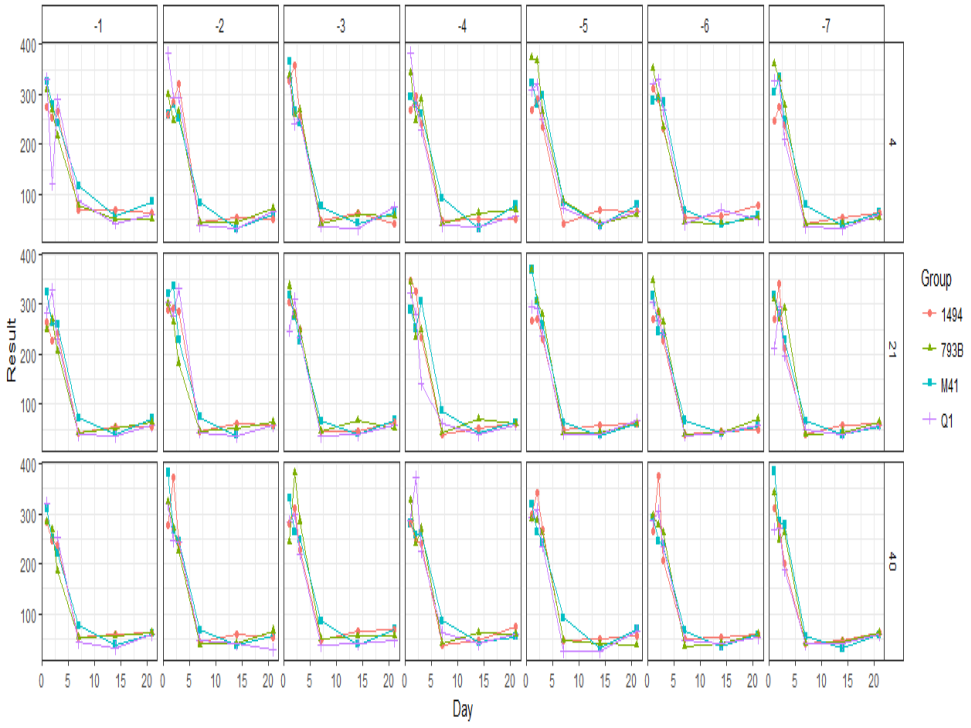

Figure 3.7. Total RNA Nano drop, 1 mg/microliter from RNA dilution extracted at different temperatures and different time points.

3.4 Discussion

The current study aimed to establish and test a universal RT-PCR protocol for detection and sequencing of the full S1 gene of globally distributed IBV strains (M41, 793B, D274, IS/1494/06, IS/885/00, Q1 and QX). Common practice for IBV research is to extract RNA for RT-PCR and sequencing, in order to detect and genotype amplified IBV strains, which

is an effective tool for the discrimination of field strains (Kwon *et al.,* 1993; Wang and Huang 2000). Many laboratories rely on a partial S1 gene assay, but a full S1 gene assay targeting a number of common IBV serotypes has not been reported to date. One limitation is that conserved sequences of approximately 20 nucleotides within the S1 gene are limited among IBV strains, decreasing the availability of applicable universal oligonucleotides (Adzhar *et al.,* 1996). Additionally, the G+C ratio of a sequenced region and the primers used can affect both reverse transcription and PCR amplification (Dohm *et al.,* 2008; Sendler *et al.,* 2011). However, the ratio in our amplification primers (A and 22.51) (36% and 47% respectively) are similar to those used previously (Adzhar *et al.,* 1996).

The full S1 protocol described in this study gave a lower average nucleotide homology between genotypes when compared to the partial S1, demonstrating that full S1 sequencing is a more accurate method to differentiate between strains using BLAST and phylogeny analysis. This is partly due to a single nucleotide variation affecting 0.3% homology for a partial sequence (350bp), compared to 0.06% for a full gene sequence (1,600bp). In addition, virulence properties can be altered through minimal amino acid variations (Fang *et al.,* 2005), although such alterations may also occur outside of the S1 gene (Callison *et al.,* 2001). IBV genotyping is commonly conducted using a partial sequence containing a hypervariable region (HVR) of S1. However, such an approach shows inconsistencies compared to full S1 genotyping (Li *et al.,* 2012; Mo *et al.,* 2013). Significantly, our phylogenetic assessment demonstrated that both the full and partial sequencing were able to distinguish individual genotypes. However, it should be noted that relying solely on partial sequences for phylogeny may give a false interpretation, depending on the sequenced region used for analysis. The full gene sequence would give a better representation of strain differences (Valastro *et al.,* 2016). Due to this, using the full S1

sequence when possible would be advantageous for studies utilising this genotyping methodology.

Not all laboratories are able to isolate and propagate IBV. Instead a number of countries are dependent on utilising FTA cards to transport potential IBV positive samples (such as tracheal or oropharyngeal swabs, lung tissue, caecal tonsil and kidneys) for diagnosis, as the cards are a safe method of storing and shipping genomic material (Ganapathy *et al.*, 2015). For IBV, it has been shown that RNA can be successfully extracted from samples embedded onto FTA cards, and following RT-PCR and sequencing, genotypes can be determined based on the partial S1 gene (Abdel-Moneim *et al.*, 2006; Savage *et al.*, 2009; Ganapathy *et al.*, 2015; Ball *et al.*, 2016c). Moscoso and colleagues (2005) and Awad and colleagues (2014) also demonstrated the effects of different temperature storage of IBV inoculated FTA cards (Moscoso *et al.*, 2005; Awad *et al.*, 2014). To date, there has been no published attempt to extract and sequence full S1 genome from samples embedded onto FTA cards outside ours.

For RNA extracted from allantoic fluid or FTA cards, the partial S1 RT-PCR demonstrated greater detection limits compared to the full S1. This may be due to the significant difference in amplicon length, about 0.39kb and 1.72kb for the partial and full S1 gene respectively. Successful molecular characterization of small sequence lengths (383 bp) of viral RNAs from FTA cards, including those that were stored under non favourable environmental conditions (at 41^{o}C for at least 15 days), has been previously demonstrated(Moscoso *et al.*, 2005). Our work has shown that the partial assay was able to detect IBV RNA inoculated onto FTA cards up to 21 dpi for all storage conditions (including 40°C). However, this was not possible for the full S1 gene (1,700 bp). From data presented here, the sensitivity for the larger target (full S1) detection is affected by both

time and temperature. This finding is in agreement with a recent study which had difficulty in amplifying a product greater than 900 bp, despite detecting a 290 bp product after six months of storage (Sakai *et al.*, 2015). In our study, an attempted to mimic field practices, where oropharyngeal swabs or tissue samples collected at necropsy or IBV-enriched allantoic fluids are embedded onto FTA cards, stored, and/or transported at different temperatures to local or international laboratories for detection of IBVs. It appears that such samples are suitable for partial S1 RT-PCR and sequencing for IBV genotyping, but provides insufficient RNA for full S1 RT-PCR or sequencing and so is unadvisable from these results.

The full S1 scheme presented in this study may be more applicable to IBVs already successfully enriched (e.g. SPF eggs or TOCs). Clinical samples (either swabs or tissues) that have been directly obtained from the field or *in vivo* experiments, including those imprinted on FTA cards, are unlikely to provide sufficient genome quantities for the full S1 detection.

Chapter 4: Pathogenesis and innate immune responses of infectious bronchitis virus Q1 in specific pathogen free chicks

Abstract

The IBV Q1 serotype was first isolated from China in the 1990s; however, there still remains a knowledge gap in the immunopathogenesis and tissue tropism of this now widespread variant. Following Q1-like infection at day-old, specific pathogen free (SPF) chicks were monitored daily for clinical signs and body weights were recorded at weekly intervals. At 1, 3, 7, 14, 21 and 28 days post infection (dpi), oropharyngeal (OP) and cloacal (CL) swabs were used for virus detection, blood was taken from ten chicks and five birds were humanely killed for necropsy. Tissue lesions were scored and trachea, kidney, and proventriculus samples were collected for RT-PCR, quantitative RT-PCR (qRT-PCR) and histopathology. Infected chicks had a significantly reduced body weight compared to the control group at 14, 21 and 28 dpi. Clinical signs of râles and nasal discharge developed in the infected group, which were absent in the control birds. All swabs were RT-PCR positive and the IBV viral load peaked in the trachea and kidneys at 9 dpi, whereas the proventriculus peaked at 14 dpi. At 28 dpi, 63% of birds were IBV-antibody positive by ELISA. We found a significant up regulation in the expression of several genes (IFNα, TLR3, MDA5, LITAF, IL1-β and IL-6) in the trachea and kidneys at 3, 7 and 9 dpi. Findings show that this Q1 isolate is pathogenic to SPF chicks with more severe lesions affecting the trachea compared to the kidneys.

4.1 Introduction

The emergence of new infectious bronchitis virus (IBV) serotypes/genotypes may have been influenced by persistent genetic mutations and recombination events (Cavanagh 2007). A number of these variants have economic importance due to their antigenic and pathogenic differences, and a lack of vaccine cross protection. While the respiratory tract is the main site of infection, non-respiratory organs such as the kidneys and proventriculus can also be infected, depending on the IBV strain (Ganapathy *et al.,* 2012).

In the period between 1996 and 1998, a new IBV variant was detected in Chinese layer flocks. This was later categorised as the Q1 genotype. Infection was associated with respiratory distress, proventriculitis, decreased egg production and diarrhoea. Sesti (2014) inoculated 25-day old broilers with Q1. The broilers presented with high mortality three days later, accompanied by severe septicaemia and nephritis (Sesti 2014). As acute and severe clinical signs are commonly shared with other pathogens (such as Newcastle disease and avian influenza virus), it was suggested that proventriculitis is not definitive evidence of a Q1 IBV infection (Sjaak de Wit *et al.,* 2011), and further characterization was required for confirmation (Liu *et al.,* 2009).

Several Q1-like IBV strains have been reported in countries such as Taiwan (2005), Italy (2011) (Toffan *et al.,* 2013), Chile (2009), Peru and Argentina (Sesti 2014), and Colombia (Jackwood 2012). According to the findings of Toffan et al., (2013), the detected Q1 strain had 100% homology with the original Q1 described by Yu et al., (2001), and (99.2%) with CK/CHC/CLDL/97 (Toffan *et al.,* 2013), which was previously reported from three countries in the Middle East (Iraq, Jordan, and Saudi Arabia) (Ababneh *et al.,* 2012). A further study showed that Q1 represented around 11% of the genotypes circulating in this area, specifically in Saudi Arabia (Ganapathy *et al.,* 2015).

Key components of the innate immune response in chickens include pattern recognition receptors (PRRs). The cytoplasmic surfaces of host immune cells express PRRs, which include lymphocytes, macrophages, dendritic cells and some non-immunological cells such as fibroblasts, and endothelial cells (Chhabra *et al.,* 2015a). These cells rapidly identify infective pathogens through PRRs such as retinoic acid-inducible gene 1 (RIG-I) like receptors (RLRs), nucleotide-binding oligomerisation domain (NOD) like receptors (NLRs) and Toll-like receptors (TLRs). Toll-like receptors are considered the central mediators of the innate immune system and are responsible for recognising conserved structures in a wide-range of pathogens (Chhabra *et al.,* 2015a). The role of melanoma differentiation associated protein 5 (MDA5) is well established in the induction of interferon (IFN) transcription via MDA5-dependent activation of the IFN-β (Kint *et al.,* 2015). Most cells have a common membrane receptor that belongs to the type I IFN family. The receptor comprises of dual subunits (IFNAR1 and IFNAR2), which has antiviral properties (Domanski *et al.,* 1995). Type I IFNs demonstrate prominently individual anti-proliferative, anti-viral and pro-apoptotic actions despite sharing similar receptors (Foster *et al.,* 1996). The IFN stimulated genes are the crucial effectors of IFN cellular responses and are responsible for their immunomodulatory, anti-proliferative and anti-viral purposes (Schoggins *et al.,* 2011).

Pro-inflammatory cytokines, such as interleukin-1β (IL-1β), interleukin-6 (IL-6) and lipopolysaccharide-induced tumor necrosis factor (TNF)-α (LITAF) are important aspects of innate immunity. They are fundamental regulatory elements that bind to specific cell surface receptors to initiate cascades of intracellular signalling (Kotani *et al.,* 2000). The IL-1β molecule is produced by epithelial cells and macrophages, which induce T cell and macrophage activation, recruiting cells to the site of infection by chemotaxis (Babcock *et al.,* 2008). IL-6 is produced by endothelial cells, T cells and macrophages, and promotes

acute phase responses and differentiation of both B and T cells (Gabay 2006). TNF-α is produced by T cells, macrophages and NK cells, and helps localise inflammation and endothelial cell activation at the site of infection, which permits greater access of other mediators such as antibodies and immune cells (Gruys *et al.,* 2005).

To date, published information on the pathogenesis and innate immune responses of Q1 virus has been limited. We aimed to address this by investigating the pathogenesis and tissue distribution of IBV Q1 in SPF chicks. In addition, the host innate immune response to Q1 in the trachea and kidneys was examined.

4.2 Materials and methods

4.2.1 Chick welfare and management

Commercially available fertile SPF eggs were incubated and hatched at the University of Liverpool (Chapter 2.1). Chicks were kept up to 28 days of age in an isolation unit (Block B) throughout the experiment and reared on deep litter with antibiotic-free water and feed (Chapter 2.4).

4.2.2 Infectious bronchitis virus

The IBV Q1 virus strain (reference number 37089/2013) (Chhabra *et al.,* 2015a) was propagated in embryonated SPF eggs(Chapter 2.1) , with virus-rich allantoic fluid collected and titrated in trachea organ cultures (TOCs) to determine a relative titre of $10^{6.5}$ CD_{50}/ml (Chapter 2.2).

4.2.3 Experimental design

Eighty SPF chicks were allocated in two groups; Group (1) was mock-infected with virus free allantoic fluid, group (2) was infected oculonasally with $10^{4.5}$ CD_{50}/bird of IBV Q1. Clinical signs were observed daily and 10 randomly picked chicks from each group were weighed weekly. Oropharyngeal and cloacal swabs were collected from ten birds at 0, 3, 7,

9, 14, 12, 28 days post infection (dpi). At 1, 21, 28 dpi, blood was collected and sera were subjected to ELISA. At 1, 3, 7, 9, 14, 21, 28 dpi, five chicks were humanely killed with the trachea and kidney scored for gross lesions. Tissues of trachea, kidney and proventriculus were collected in RNAlater (Qiagen, Crawley, UK) for real time PCR analysis (trachea and kidney) and in formalin and OCT media (all tissues) for histological examination (Flaño *et al.*, 2009).

4.2.4 Swab samples

Dry swabs were used to sample the cloaca and oropharyngeal cavity at 3, 7, 9, 14, 21, and 28 days post infection. After sampling, swabs from each group were pooled, dipped into 1.5 ml of TOC media, and stored at -70^{o}C until required.

4.2.5 Clinical observations

Clinical signs will were recorded daily according to a previously published scoring system (Grgic *et al.*, 2008) (Chapter 2.8.1). Gross lesions recorded as previously described (Mahgoub *et al.*, 2010)(Chapter 2.8.2).

4.2.6 Histopathology

The tracheal upper part and the superior kidney part were collected and fixed in 10% buffered formalin were processed for histological examination (Chapter 2.11). The proventriculus was processed following storage in frozen OCT media (Flaño *et al.*, 2009), (Chapter 2.11).

4.2.7. Extraction of RNA

4.2.7.1 Swabs, and allantoic fluid

RNA was extracted from OP and CL swabs, and allantoic fluid (AF) using the phenol-chloroform method (Chapter 2.12) (Chomczynski and Sacchi 2006; Ball *et al.*, 2016a). All the swabs sequencing cleaning and alignment details are present in Chapter 2.17.

4.2.7.2 Extraction of RNA (Tissue)

Total RNA was extracted from all collected tissues using the the RNeasy Plus Mini Kit following the manufacturer's instruction RNA (Qiagen) according to manufacturer's instructions (Chapter 2.14).

4.2.7.3 Reverse transcriptase polymerase chain reaction (RT-PCR)

Following extraction, RT-PCR to detect a partial IBV S1 sequence (393bp) was conducted on swabs and allantoic fluid as previously described (Ball *et al.*, 2017), (Chapter 2.15).

5.2.8 Quantification of IBV RNA

qRT-PCR Viral load was quantified from extracted RNA from trachea, kidney, proventriculus and OP and CL swabs (Chapter 2.18).

4.2.9 Tissue samples for virus isolation and RT- PCR

Sampled tissues were ground aseptically using a pestle and mortar and 300µl of TOC media. Samples were stored at -70 ^{o}C until processed for RNA extraction.

4.2.10 Virus isolation from proventriculus tissue

Virus isolation was attempted from proventriculus samples taken at 3, 7 and 9 dpi (Yu *et al.*, 2001; Toffan *et al.*, 2013). Ground tissues were centrifuged and 0.2 ml from the supernatant was inoculated into embryonated SPF eggs (Chapter 2.10).

4.2.11 Host gene expression

Host gene expression analysis was carried out for samples collected at 1, 3, 7, 9, and 14 dpi. The details of qRT PCR host genes and cytokines (Chapter 2.19). QRT-PCR of cDNA samples was performed in triplicate using LightCycler 480 SYBR Green I Master mix and primers (Chapter 2.19).

4.2.12 Serology

Serum samples were analysed using a commercial IBV ELISA kit (IDEXX) according to manufacturer's instructions (Chapter 2.20).

4.2.13 Immunohistochemistry

All the details of Cryostat (tissue sections) and staining of slides from the proventriculus (Chapter 2.21).

4.2.14 Statistical analysis

All statistical analysis was carried out in GraphPad Prism version 6 using the t test to make comparisons between the groups. Differences between groups were considered significant at $P<0.05$ (Chapter 2.22).

4.3 Results

4.3.1 Body weight

There was no significant difference in body weight between the control and Q1-infected group in the first week. After 14 dpi, we saw a significant decrease ($P<0.05$) in the average weekly body weight of chicks in the infected group at 14, 21 and 28 dpi (Figure 4.1).

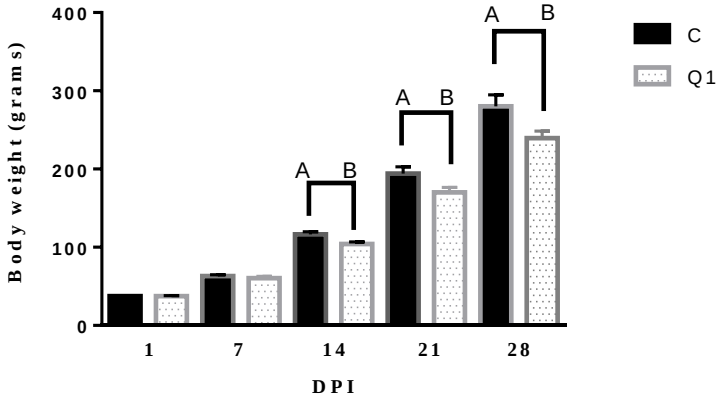

Figure 4.1. Weekly mean body weight comparison of control (C) and infected (Q1) on all sampling days. Data represents the mean with error bars as standard error with significant differences indicated by different letters (P<0.05).

4.3.2 Clinical signs

Mild to moderate clinical signs appeared within infected birds at 24 dpi and continued until 17 dpi. Signs mainly composed of sneezing, râles and coughing and nasal discharge (Figure 4.4A). A single bird from the infected group died at 5 dpi and on necropsy presented with swollen kidneys with ureters filled with urate. There were no clinical signs or mortality in the control group during the study period (Figure 4.2).

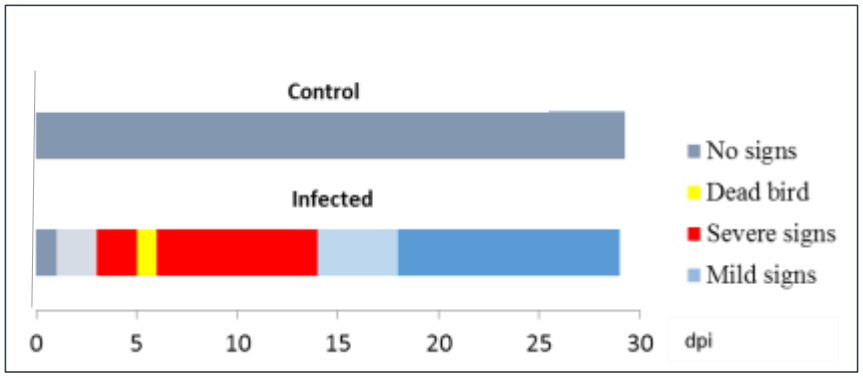

Figure 4.2. Shows the onset and duration of clinical signs and mortality within the period of the experiment (28 days) in controls and IBV Q1 infected group.

4.3.3 Gross lesions

Tracheal lesions were noted in all infected chicks until 14 dpi, peaking at 7 dpi (Figure 4.3). Lesions comprised of clear to caseous exudate with or without hyperaemia (Figure 4.4B). Birds showed signs of oculo-nasal discharge and periocular swelling at 14 dpi, with cloudy and turbid air sacs found at 9 dpi. In the kidney, gross lesions consisted of paleness, swelling and urate deposition. The highest mean lesion score in the kidneys was at 9 dpi. In the proventriculus, there was dilation and thickening in the mucosa of one bird at 14 dpi (Figure 4.4D). There were no gross lesions observed in the control group.

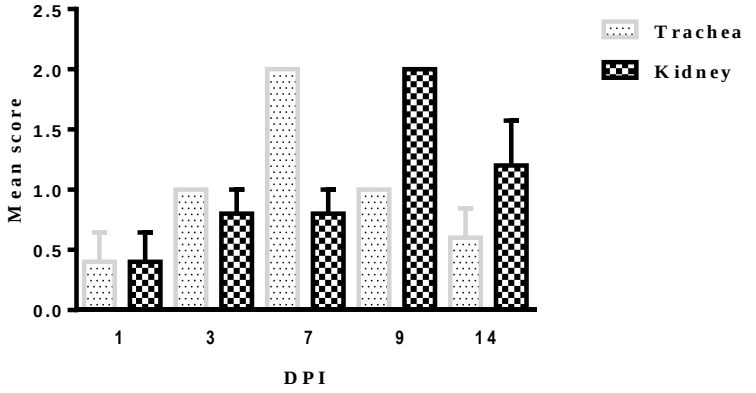

Figure 4.3. Gross lesion score in the trachea and kidney of Q1 infected SPF birds. Data represents the mean with error bars as standard error.

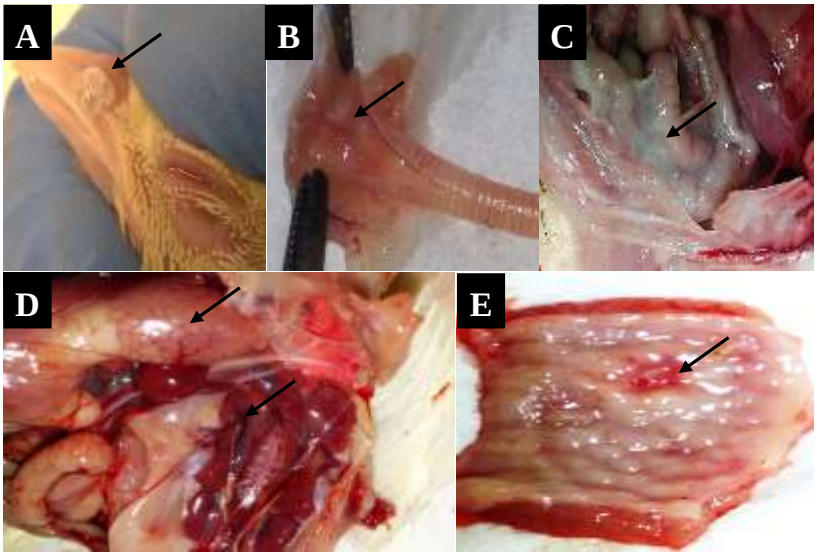

Figure 4.4. Developing of the gross lesion after IBV Q1 oculo-nasal inoculation into SPF chicks at one day old. (A). Chick with watery to frothy oculo-nasal discharge at 7 dpi (B). Trachea revealed congestion at 7 dpi (C). Cloudy and turbidity yellowish thoracic air sac developed SPF chick after infection with IBV Q1 at 9 dpi (D). Swelling kidneys with nephritis and deposition of uric acid in ureters and proventriculus dilation in SPF chick after 14 days of infection (E). Proventriculus ulcer and haemorrhage in the mucosa at 14 dpi.

4.3.4 Histological changes

In the Infected group, tracheal lesions mainly consisted of de-ciliation and epithelial degeneration, decreased mucous cells and heterophil Infiltration (Figure 4.6B). Lesions started early at one dpi and remained until 21 dpi. The greatest tracheal histopathology scores were seen at nine and 14 dpi (Figure 4.5A). In the kidney, histological changes started at 1 dpi and peaked at 3-9 dpi (Figure 4.5B). Kidney lesions consisted of poorly defined subacute interstitial lymphoid Infiltration and more discrete chronic lymphoid

nodules (Figure 4.6D). In the proventriculus, there were no significant microscopic lesions other than the presence of interstitial lymphoid nodules (Figure 4.6F). There were no histological changes in the control group.

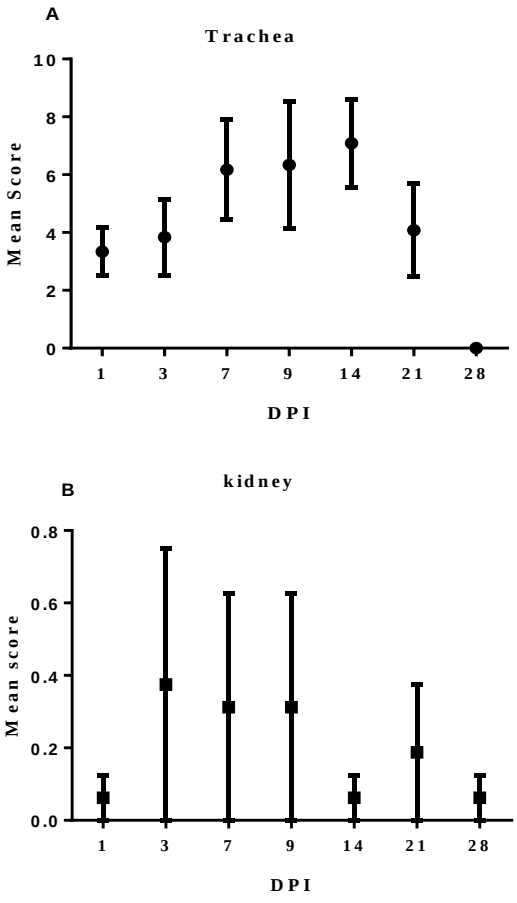

Figure 4.5. Histopathological score of lesions in the trachea and kidney of infected group (A) Trachea (B) kidney. Birds (n=5/group) were infected with IBV strains Q1 IBV at a dose of $10^{4.5}$ CD_{50}/bird and processed for histopathological examination at 1, 3, 7, 9, 14 , 21 and 28 days post infection (dpi). Data represents the mean with error bars as standard error.

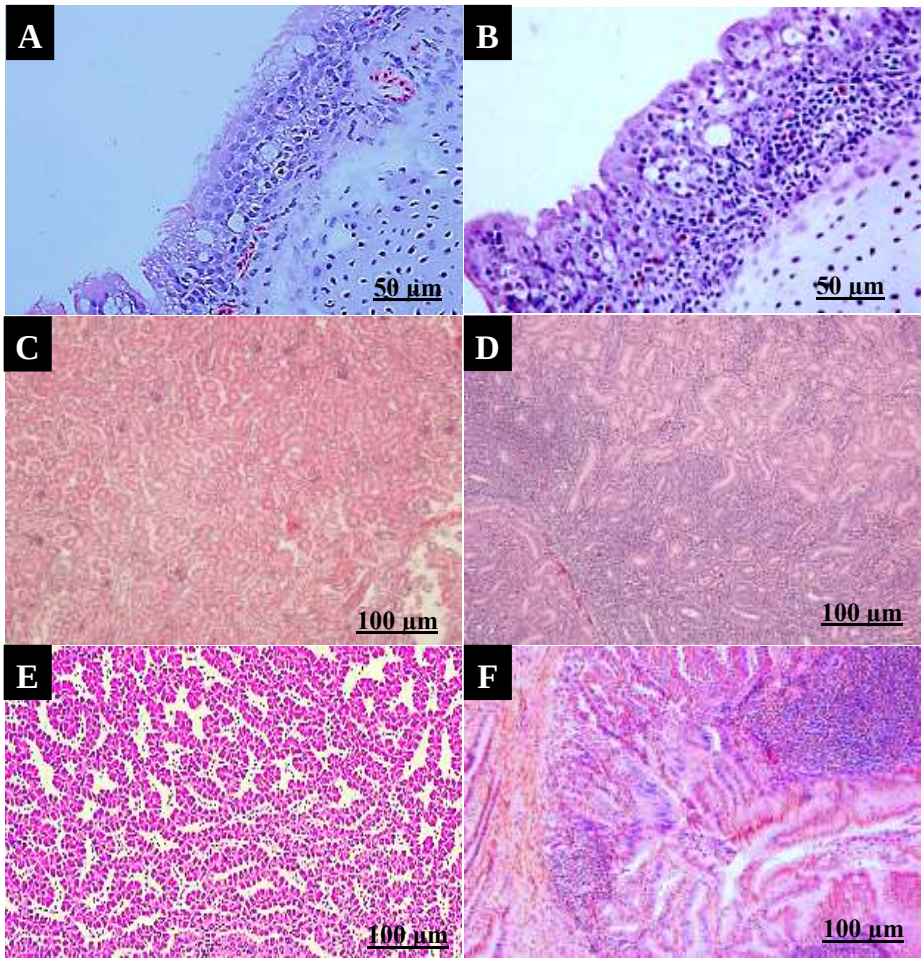

Figure 4.6. Haematoxylin and eosin stains of the controls; ((A. Trachea), (C. Kidney) and (E. proventriculus)) and Infected tissues ((B trachea at 9 dpi), (D kidney at 9 dpi) and (E proventriculus at 28 dpi)).

4.3.5 Detection of Q1 from pooled oropharyngeal (OP) and cloacal (CL) swabs

4.3.5.1 RT-PCR:

All OP and CL swabs were positive by RT-PCR, with partial S1 gene sequences obtained at all sampling points. Amino acid (AA) sequences from OP swabs remained identical to the original inoculum at 3, 7, 14 and 28 dpi. For OP samples collected at 9 dpi, there were 22 AA substitutions. At 21 dpi there were 15 AA substitutions and two AA deletions: tyrosine(Y) and asparagine (N) at 919 nucleotide positions within S1 gene). By 28 dpi, the recovered strains were identical to the inoculum. In the CL swabs, sequence changes presented early (3 dpi), consisting of 11 AA changes. At 7 dpi, there were 30 AA changes. At 9 dpi, there were 19 AA changes. At 9 dpi, there were also three AA deletions tyrosine(Y), asparagine (N) and phenylalanine (F) at 919 nucleotide positions within S1 gene. At 21 dpi, there were over 40 AA changes. These changes were completely absent at 28 dpi. Comparing sequences obtained from the CL swabs and the inoculum, the similarity were 94%, 83%, 88% and 80% for 3, 7, 9 and 21 dpi respectively.

4.3.5.2 qRT-PCR:

All OP and CL swabs were positive for IBV until 21 dpi. The viral load was greatest at 3 dpi for both OP and CL swabs, the quantity decreased until 14 dpi where it remained at a low level until 21 dpi (Figure 4.7). Overall, there was a greater viral load in the OP swabs compared to the CL swabs for all time points.

Table 4.1. Sequence alignment of the S1 glycoprotein of IBV Q1 strain from OP and CL swabs at 3, 7, 9, 14, 21 and 28 dpi. Dots indicate identical amino acid to the original inoculum (first row of aligned sequences). Letters denote change in the amino acid. Dashes denote deleted amino acids.

OP swabs

	VNTTLVLTNF	TFSNVSNAPP	NTGGVHSIVL	HQTQTAQSGY	YNFNFSFLSS	FRYVESDFMY	GSYHPKCSFR	LETINNGLWF	NSLSVSLGYG	PLQGGCKQSV
OP 3	....	....	....	....	....	....	....	....	....	....
OP 7	....	....	....	....	....	....	....	....	....	....
OP 9	....	....	....	....	.L......IZ	CSK.Y....P	PPNLL..TIK	QCS.MDD.C.	..F.....AT	R.........
OP 14	....	....	....	....	....	....	....	....	....	....
OP 21	....	....	....	....	--.......R	...G.A....	R.D.HRQ.C.	........GL	S.......AH	..P.......
OP 28	....	....	....	....	....	....	....	....	....	....

CL swabs

	VNTTLVLTNF	TFSNVSNAPP	NTGGVHSIVL	HQTQTAQSGY	YNFNFSFLSS	FRYVESDFMY	GSYHPKCSFR	LETINNGLWF	NSLSVSLGYG	PLQGGCKQSV
Cl 3	....	....	....	....	....	.S........	..NL...TIK	Q.C.......	..F....A..	R.........
CL 7	....	....	....	....	.LL.....IC	CSK.Y....P	PPNLL..TIK	Q.CFVVD.C.	..F.F..PAT	R.........
CL 9	....	....	....	....	---I.....F	...E.TY..S	..N.Q....K	.QV...S...V	...FLF.VD.	..E.......
CL 14	....	....	....	....	....	....	....	....	....	....
CL 21	.I...K....	....	....	....	FLLS.Y..IC	LIK...V..P	PTKLL.TTNK	..C.VVD.C.	SFFWLPPAT.	R..AV.INT.
CL 28	....	....	....	....	....	....	....	....	....	....

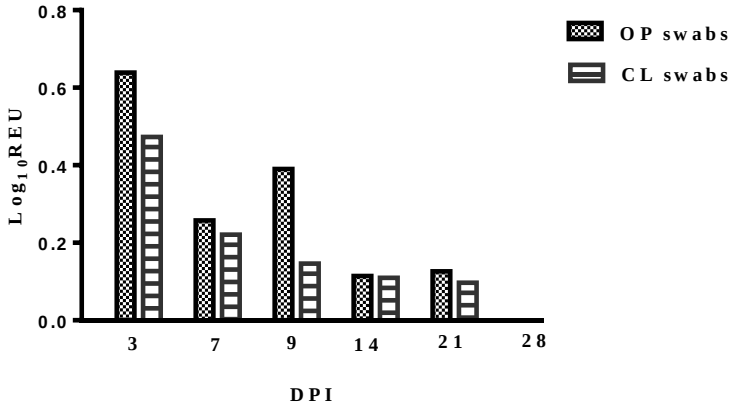

Figure 4.7. Quantification of viral RNA, expressed as a log relative equivalent units (REU) of RNA in the pooled OP and CL swabs from the Q1 infected group.

4.3.5.3 Viral load in trachea, kidney and proventriculus

Viral RNA was detected in all tissues for all sampling days; however, each tissue exhibited a different expression pattern (Figure 4.8). Viral load peaked in the trachea at 7 dpi (0.533 Log 10 REU), kidneys at 9 dpi (0.121 Log 10 REU) and proventriculus at 14 dpi (0.202 Log 10 REU) (Figure 4.8A, B and C). Following this, viral RNA load decreased in all infected tissues to the lowest level at 28 dpi. In the trachea, there was significant higher differences ($P<0.05$) at 1-14 dpi when compared to 21-28 dpi.in the kidney, there, there was significant higher differences ($P<0.05$) at 1-21 dpi when compared to 28 dpi.

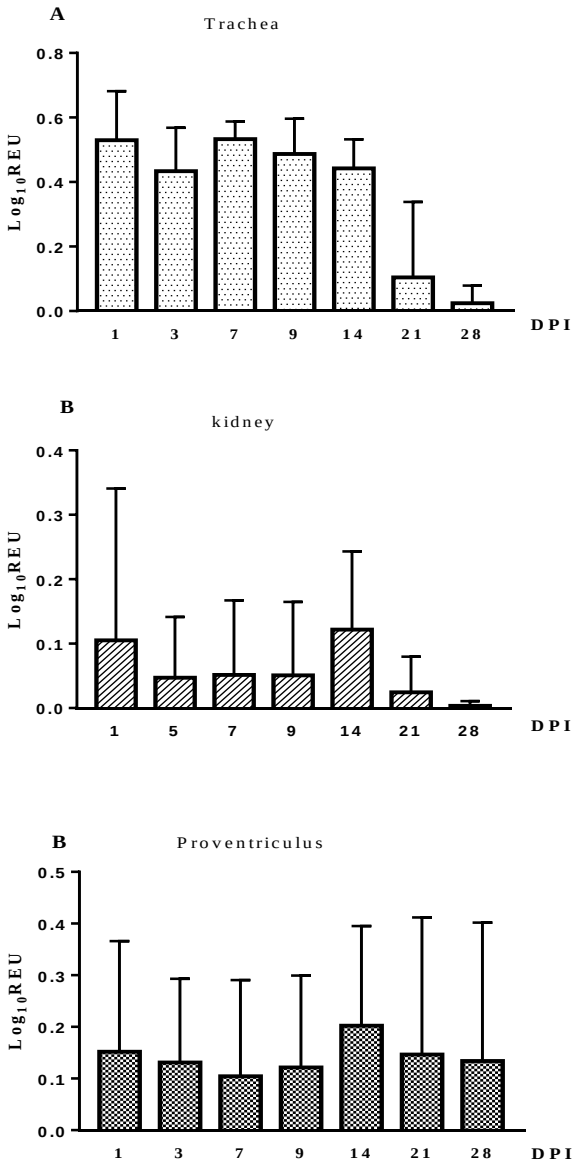

Figure 4.8. Quantification of viral RNA, expressed as a log relative equivalent units (REU) of RNA in (A) Trachea, (B) Kidney and(C) proventriculus of the Q1 infected group. Data represents the mean with error bars as standard error.

4.3.6 Isolation and immunohistochemistry detection of IBV from the proventriculus

4.3.61 Virus isolation

Virus re-isolation was attempted from the proventriculus at 3, 7, 9 dpi in embryonated chicken eggs (ECE). The virus was detected from all samples at all-time points, and on sequencing, the isolates had > 99% amino acid similarity to the inoculum. No virus isolation attempted from other tissues.

4.3.6.2 Detection of viral antigen using immunohistochemistry

In the proventriculus mucosa, IBV antigen was not detected and also from the proventriculus smooth muscle. In other words, there was no positive immunostaining in all proventriculus tissue (Figure 4.9).

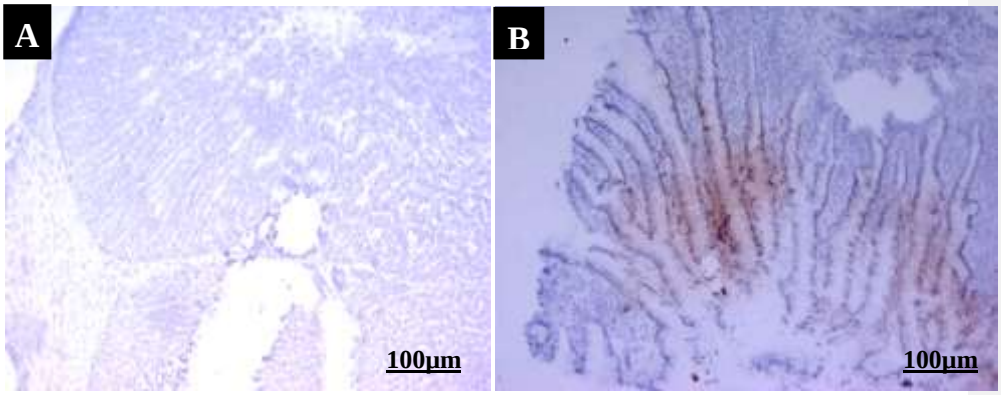

Figure 4.9. Immunohistochemically detection of Q1 IBV antigens in Proventriculus. (A) Control uninfected group. (B) Infected group, proventiculus negative staining, there is no immunolabelling of smooth muscle cells and within the vasculature cells at 7 dpi.

4.3.7 Humoral anti-IBV antibody levels

Serum samples at one dpi showed no positive antibodies against IBV in either the control or infected groups. At 21 dpi, 43% of infected birds were ELISA positive against IBV which had increased to 63% by 28 dpi (Figure 4.10).

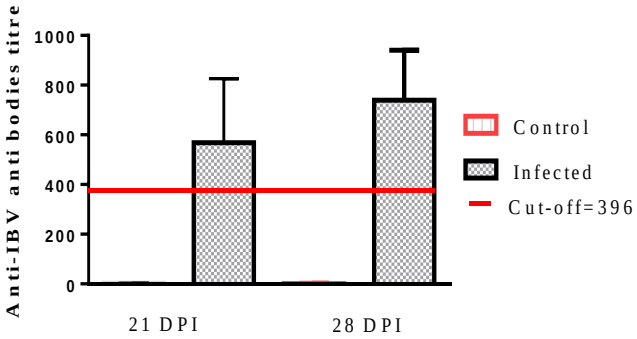

Figure 4.10. Mean infectious bronchitis virus (IBV) antibody titres of the control and infected groups (n=10/group) at 21 and 28 days-old. Data are expressed as mean values ± SEM (N=10). The ELISA positive sample cut-off is 396.

4.3.8 Host gene expression analysis in the trachea

4.3.8.1 Relative IFN-α and IFN-β mRNA expression:

There was significant up–regulation ($P<0.05$) in the IFN-α mRNA expression at 7 dpi compared to 3 dpi, no other changes were found in the subsequent sample points (Figure 4.11A). There was significant up-regulation in IFN-β mRNA expression at 1 and 3 dpi only (Figure 4.11B).

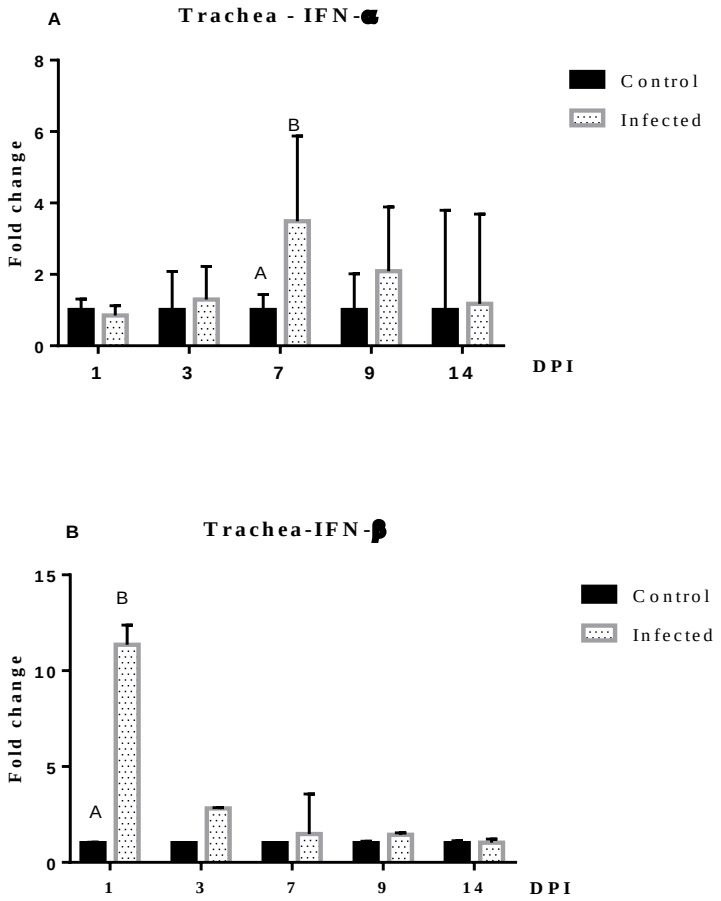

Figure 4.11. mRNA expression of interferon (IFN I) (A) IFN-α and (B) IFN-β mRNA expression in Q1 infected chicken trachea. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters ($P<0.05$).

4.3.8.2 Expression of mRNA of TLR3 and MDA5:

There was significant up-regulation in TLR3 expression ($P<0.05$) for all sampling days compared to the control group (Figure 4.12C). There was significant up-regulation ($P<0.05$) seen at 3 dpi of MDA5 (Figure 4.12D).

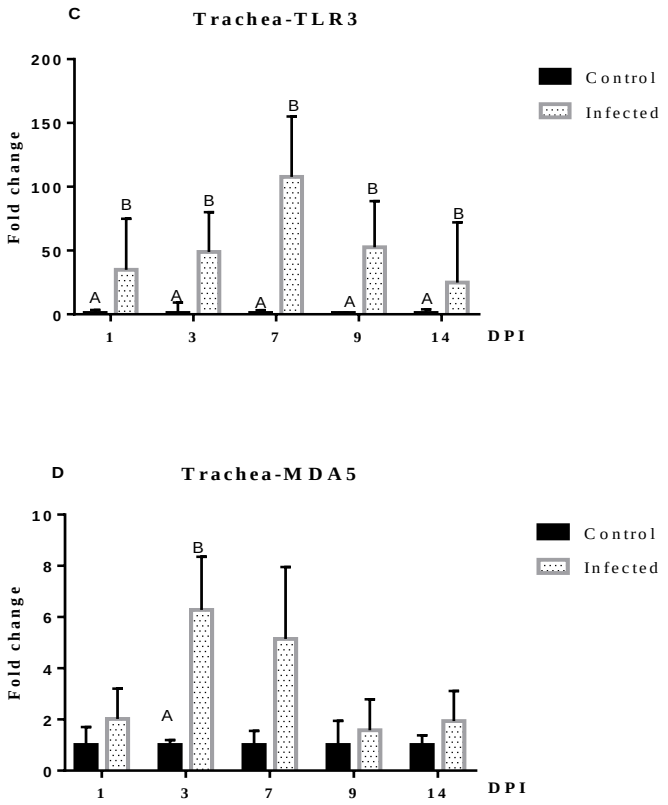

Figure 4.12. Transcriptional regulation of innate viral sensing molecules (C) TLR3 and (D) MDA5 in Q1 infected chicken trachea. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters ($P<0.05$).

4.3.8.3 Pro-inflammatory cytokines transcription profile:

Expression of IL1-β was significantly up-regulation ($P<0.05$) at 7 dpi in Infected group compared with control group (Figure 4.13 E). The IL-6 gene was up-regulated at all sampling points (significant at 7 dpi; $P<0.05$) (Figure 4.13F). Similarly, there was significant up-regulation ($P<0.05$) in the LITAF for all sampling days (Figure 4.13G).

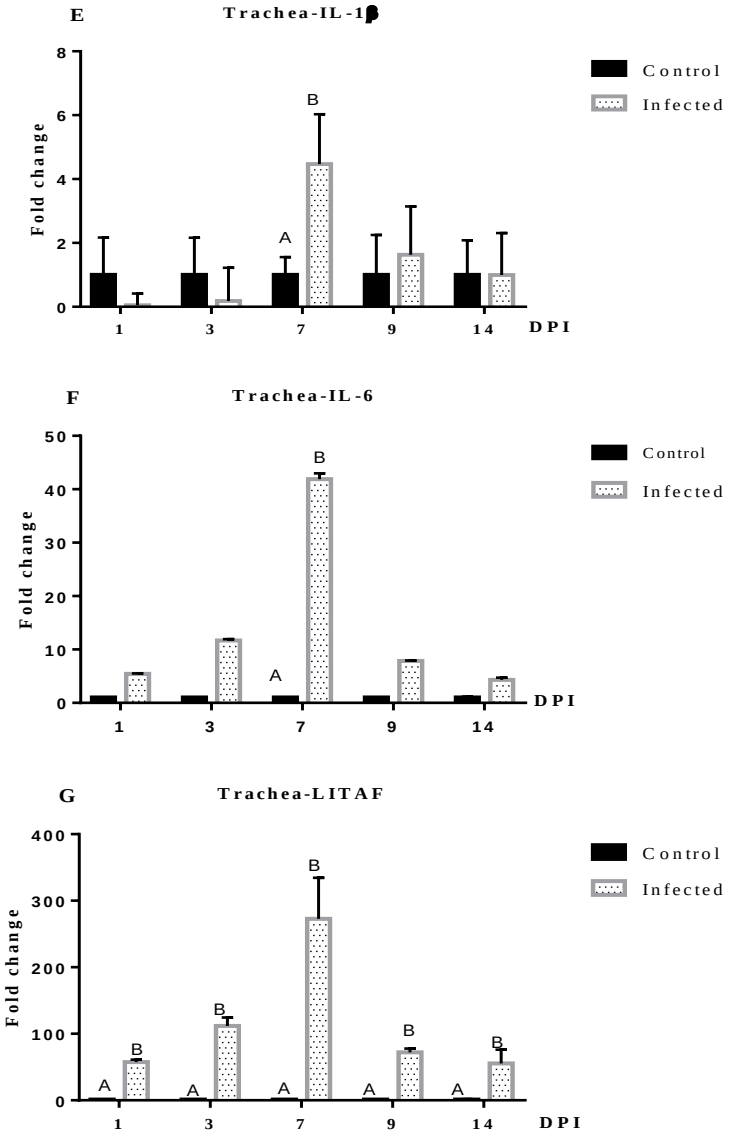

Figure 4.13. Transcription profile of proinflammatory cytokines (E) IL-1β, (F) IL- 6 and (G) LITAF in Q1 infected chicken trachea. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters ($P<0.05$).

4.3.8.4 Host gene expression analysis in the kidney

4.3.8.4.1 Relative IFN-α and IFN-β mRNA expression:

There was significant ($P<0.05$) up-regulation identified in IFN-α expression levels at all dpi in the Infected group compared with the control group (Figure 4.14A). Similarly, there was significant up-regulation ($P<0.05$) of IFN-β mRNA expression in the kidney samples from the Infected group compared with the uninfected control group for all sampling days (Figure 4.14B).

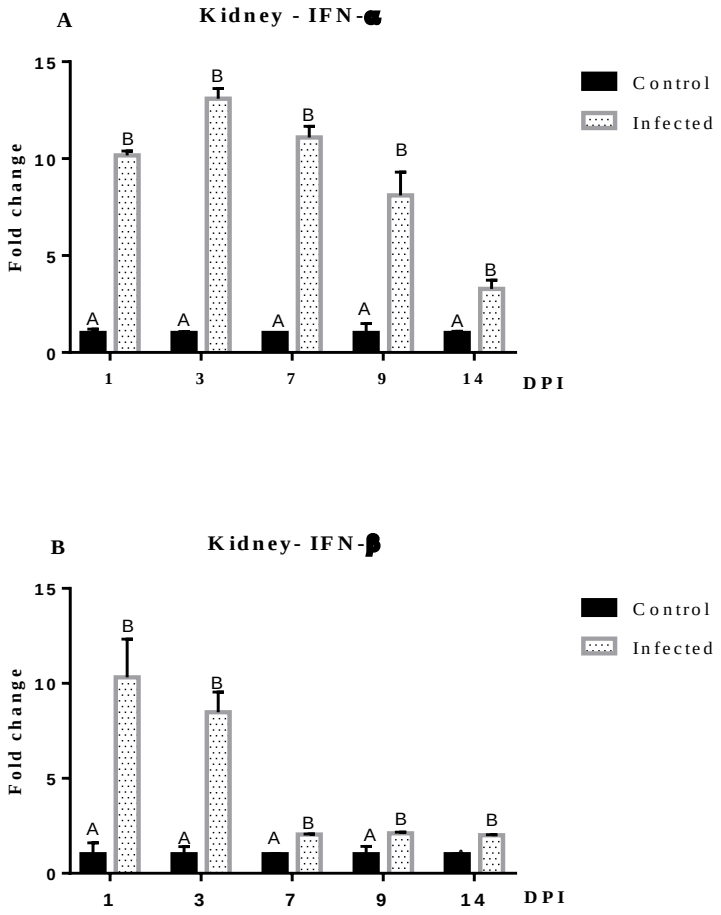

Figure 4.14. mRNA expression of interferon (IFN I) (A) IFN-α and (B) IFN-β mRNA expression in Q1 infected chicken kidney. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters ($P<0.05$).

4.3.8.4.2 Expression of mRNA of TLR3 and MDA5:

There was significant up-regulation in TLR3 expression ($P<0.05$) for all sampling days compared to control group (Figure 4.15C). There was significant up-regulation ($P<0.05$) in MDA5 for all sampling days in comparison to the control group (Figure 4.15D).

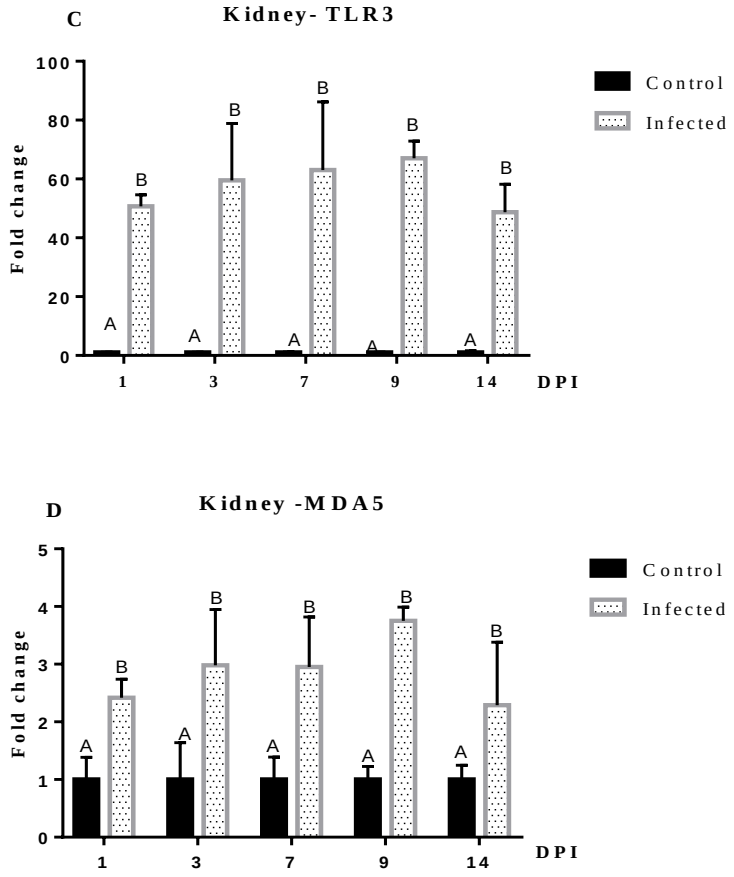

Figure 4.15. Transcriptional regulation of innate viral sensing molecules (C) TLR3 and (D) MDA5 in Q1 infected chicken kidney. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters ($P<0.05$).

4.3.8.4.3 Pro-inflammatory cytokines transcription profile:

There was significant up-regulation ($P<0.05$) in IL1-β at 3-9 dpi in the Infected group compared with control group (Figure 4.16E). There was significant up-regulation ($P<0.05$) in IL-6 at 7-9 dpi in the Infected group compared with control group (Figure 4.16F). There was significant up-regulation ($P<0.05$) in the LITAF for all sampling days (Figure 4.16G).

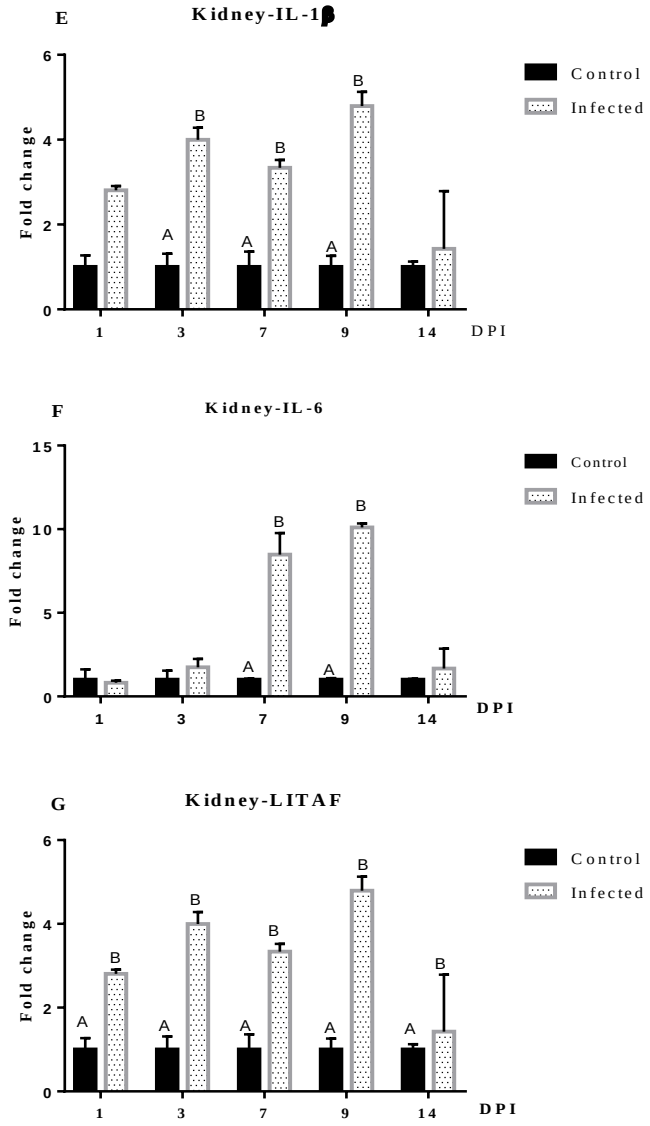

Figure 4.16. Transcription profile of proinflammatory cytokines (E) IL-1β, (F) IL- 6 and (G) LITAF in Q1 infected chicken kidney. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant changes indicated with different letters ($P<0.05$).

4.4 Discussion

Recently, the pathogenesis and/or the innate immune response of other IBV strains have been published (Awad *et al.,* 2016; Najafi *et al.,* 2016; Okino *et al.,* 2017). However, little Information has been published on the Q1 virus. For the current study, in addition to clinical signs, gross and histopathological lesions, attempts were made to examine host-virus interactions, including the induction of innate and humoral immune responses.

A significant decline in the average weekly body weight was found in Q1 infected chicks from 14 dpi. Reduction in body weight following IBV infection has been previously reported for a number of strains, including the Australian T-strain (Afanador and Roberts 1994), QX (Ganapathy *et al.,* 2012) and M41 (Otsuki *et al.,* 1990). This may be due to damage to the kidney tubular epithelial cells (Condron and Marshall 1986), leading to defects in electrolyte and fluid transport. An increase in urinary water excretion can also occur, leading to an increase of urine osmolality and high fractional excretion of sodium, calcium and potassium (Afanador and Roberts 1994). This is the first study to report a reduction in body weight in Q1 infected chickens.

The clinical signs and lesions reported in this study were similar to those found in commercial broiler (Hubbard naked neck) chickens with evidence of Q1 infections in Italy (Toffan *et al.,* 2013). For SPF chicks in this study, the duration of lesions and severity was consistently greater in the trachea when compared to the kidney. Similar findings have been reported for other IBVs such as M41 (Butcher *et al.,* 1990), QX (Ganapathy *et al.,* 2012), It-02 (Dolz *et al.,* 2012), 793B (Boroomand *et al.,* 2012), Moroccan G strain (Ambali and Jones 1990) and more recently, for IS/885/00 (Awad *et al.,* 2016). In one bird, there was thickening in the proventricular mucosa and proventricular dilation, similar to previous report of Yu et al. (2001). However, in contrast to the findings in this study, Yu

and colleagues witnessed a higher mortality rate (Yu *et al.,* 2001), which may have been due to their higher inoculation dosage, their smaller sample size or the age of the birds.

High severity of histological changes in the trachea was seen at 9 and 14 dpi, while we witnessed lower overall lesion scores in the kidney. Trachea changes manifested as loss of cilia, heterophil infiltration, epithelial hyperplasia and lymphoid infiltration, as also seen previously in Q1-like infected SPF chicks (Yu *et al.,* 2001). Kidney samples showed interstitial lymphoid infiltration and mild lymphoid follicles similar to those reported before (Toffan *et al.,* 2013). There were minimal changes in proventriculus samples, comprising of lymphoid follicles. This result is dissimilar with the prior report by Toffan et al. (2013), which found diffuse epithelial necrosis, associated with infiltration of heterophils, lymphocytes and plasma cells in the lamina propria of commercial broiler chickens. The differences could have been contributed by exacerbating factors such as co-infection with other pathogens, ammonia, cold, dust, type of feed and other management protocols in commercial broiler farms.

Viral load was greatest at 3 dpi and then gradually declined in the OP and CL swabs until it became undetectable at 28 dpi. It appears that excretion of virus in Q1-infected SPF chicks shared similar profiles of other known IBVs such as M41, Connecticut and Arkansas (Callison *et al.,* 2006). It has been shown that IBV replicates primarily in the tracheal and kidney tissues, with additional infections seen in the lung, liver and pancreas (Fan *et al.,* 2012). Results in the current study indicated that Q1 viral load was highest in the trachea compared to the proventriculus or kidneys. In addition, IBV Q1 was successfully re-isolated from the proventriculus, with the recovered isolate being more than 99% similar to the original Q1 inoculum, thereby further highlighting the possibility of using this tissue for IBV detection or isolation (Fan *et al.,* 2012; Ganapathy *et al.,* 2012). However, it was

not clear if the isolation indicates primary replication in this tissue or merely those found in the blood vessels.

To date, there are no studies focusing on the partial-S1 gene changes in virulent IBV strains detected in OP or CL swab samples, though reports are available on vaccine strains (van Santen and Toro 2008; Ball *et al.,* 2016a). We found a greater number of genetic changes in the CL swab sequences compared to the OP swab sequences, which may be influenced by the environment in the upper respiratory tract in comparison to the intestine (Zanin *et al.,* 2016). Since the S1 protein is associated with evasion from host immunity and viral proliferation (Montassier 2010), such mutations may be advantageous to the survival of Q1 in the gastrointestinal tract.

Findings from ELISA showed a seroconversion of 43 and 63% at 21 and 28 dpi respectively. The commercial ELISA kit contained an M41 coated antigen and despite Q1 being a variant with 77-82% nucleotide similarity (Ababneh *et al.,* 2012) with Mass, the assay detected a relatively high seroconversion at 21 and 28 dpi respectively. A higher percentage of birds may have seroconverted if a homologous or a common recombinant IBV was used as coating antigen rather than that from M41 (de Wit *et al.,* 1997; Awad *et al.,* 2016).

In recent years, much attention has been given to understand the early immune responses towards newly identified IBV isolates (Okino *et al.,* 2017; Chhabra *et al.,* 2018), as this could improve our understanding on disease mechanisms, which could ultimately be utilized for better control strategies. In this study, in order to monitor early immune responses, TLR3, MDA5, IFN-β, IFN- α**,** IL-1β and IL-6 mRNA expressions were examined following Q1 infection. For TLR3, significantly higher expression compared to the control was found at all sampling days, with the peak level at 7 dpi. This finding echoes

data seen in similar studies, which also reported up-regulation in TLR3 following IBV infection of different chicken lines, particularly during the early stage of infection (1-8 dpi) (Smith *et al.,* 2015; He *et al.,* 2016; Okino *et al.,* 2017; Chhabra *et al.,* 2018). However, the magnitude and onset of this up-regulation differs from others, probably due to variations in the IBV strain, inoculum dosage, route of administration and age of birds used (Smith *et al.,* 2015; He *et al.,* 2016; Okino *et al.,* 2017; Chhabra *et al.,* 2018).

The role of MDA5 is well established in induction of IFN-β transcription, through an MDA5-dependent activation of the IFN response (Kint *et al.,* 2015). In the current study, at 1 dpi, there was a significant up-regulation of MDA5 in the trachea, consistent with previous research linking IBV infection with an up-regulation of MDA5 expression (He *et al.,* 2016; Chhabra *et al.,* 2018). Limited reports are available on expression of PRRs against IBV in kidneys. Our study shows that significantly higher levels of TLR3 and MDA5 were found in the kidneys following Q1 infection. Working on IBV IS/885/00, Chhabra et al. (2018) reported up to a 5 fold up-regulation of MDA5, whereas we detected up to a 3 fold change in this study. It appears that the magnitude of MDA5 expression could reflect the degree of tropism of IBV strains to the kidneys (Cong *et al.,* 2013).

There was a lower magnitude of expression of TLR3 in the kidney compared to the trachea, which may be associated with a lower viral load in the kidneys. As infected cells distinguish virus components by PRRs, up-regulation may lead to early immune responses. Elevation of TLR3 is associated with an increase of inflammatory cytokines in response to viral pathogenesis and the subsequent deterioration of infected tissue through the downstream cascade antiviral state (Kameka *et al.,* 2014; Lin *et al.,* 2016). The onset and duration of TLR3 and MDA5 expression in this study differed to other studies and potentially related to the differences in the virulence of IBV strains (Okino *et al.,* 2017; Chhabra *et al.,* 2018).

It has been shown that IFN-β expression occurs at a later stage of infection, coinciding with the peak of viral replication and the complementary accumulation of double stranded RNA (Kint *et al.,* 2015). This study showed significant up-regulation of IFN-α and IFN-β in both the trachea and kidneys, similar to the results of a previous work on virulent IBV M41 in SPF chicks (He *et al.*, 2016; Zhang *et al.,* 2017). In this study, IFN-β expression was up-regulated up to 3 dpi, comparable to findings by Chhabra et al. (2018) and Okino et al. (2017) following infection with other virulent IBVs. However, the magnitude of fold changes reported by them were greater compared to the current study, possibility influenced by the virulence of the virus, dosage, route of inoculation, age and strain of birds used.

In addition to detecting up-regulation of PRRs and IFNs, findings in this study showed an up-regulation of pro-inflammatory gene expression in both investigated tissues. Such increase corresponds with gross lesion scores, the highest viral load in the trachea and OP swabs and one of the highest readings in the histological changes in the trachea, particular with peak detection at 7 dpi. This emphasises the combined effect of these cytokines along with virus load on the development of tracheal lesions. An increase in IL-1β and IL-6 was previously described after challenge with virulent M41, which was associated with the highest scores of microscopic lesions and viral load, suggesting IL-1β and IL-6 may have contributed to tracheal lesion induction (Okino *et al.,* 2014).

Following an *in vivo* experiment, Chhabra et al. (2018) demonstrated that a substantial and rapid innate immune response was induced in the kidney after infection with nephropathogenic strains of IS/885/00 and QX. These strains produced significantly greater expression of IL1-β, IL-6 and LITAF compared to the M41 strain, which reflected the tropism and virulence of these viruses to the kidney tissues (Chhabra *et al.,* 2018). IL-1β and/or IL-6 pro-inflammatory cytokines have been reported to correspond with gross

and histological lesions (Asif *et al.*, 2007; Okino *et al.*, 2014; Okino *et al.*, 2017). Tissue tropism affects viral replication and overall viral load, and the inflammatory response is secondary to this. All pro-inflammatory cytokines in the current study peaked at 3-7 dpi, which were associated with more viral load and pathology in the trachea than in the kidneys (Jang *et al.*, 2013).

In conclusion, the Q1 isolate used in this study is pathogenic to SPF chicks, has a greater pathology and induces greater early immune responses in the trachea compared to the kidneys. Following Q1 infection, increased mRNA expression was seen for TLR3, IFNs, and IL1-β, which are innate immune indicators of early host response to the infection.

Chapter 5: The immunopathogenesis of infectious bronchitis virus Q1 in slow or fast growing commercial broiler chicks

Abstract

The pathogenesis, humoral and innate immune responses of infectious bronchitis virus (IBV) Q1 was investigated in two lines of commercial broiler chicks with different growth rates. Chicks were monitored daily for clinical signs. At 3, 7, 9, 14, 21 and 28 days post infection (dpi), oropharyngeal (OP) and cloacal (CL) swabs were taken for virus detection. Renal, tracheal and proventricular tissue was collected at post-mortem for quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). Blood was taken at 7, 14, 21 and 28 dpi for IBV antibody detection. For the fast growing line (Line-A), swabs from the infected group were RT-PCR positive at all sampling days, whereas the slow growers (Line-B) were positive until 14 dpi. At 7-9 dpi, higher viral loads were found in the trachea, proventriculus and kidney of fast growers compared to slow growers. Mean IBV ELISA antibody titres in Line-B were higher than Line-A. Tracheal innate immune responses showed IFNα up-regulation only in Line-Abut IFNβ was up-regulated in both lines. For TLR3, an up-regulation was seen in Line-A up to 7 dpi and for all sampling days in Line-B. MDA5 was up-regulated in Line-A and down-regulated in Line-B at 1 dpi. In the kidneys, for Line-A birds, IFNα and IFNβ were up-regulated at 1 and 1-3 dpi respectively. There was up-regulation in TLR3 in Line-B throughout the study period but not for Line-A. MDA5 was up-regulated in both lines at 7 and 9 dpi. It appears that the immunopathogenesis of IBV Q1 infection in slow growing (Line-B) chicks was milder in terms of the inflammatory cytokines produced when compared to the fast growers (Line-A), which could be associated with the genetic differences between these breeds.

5.1. Introduction

Infectious bronchitis virus (IBV) is a globally important avian pathogen, of particular importance for intensive poultry production regions. This pathogen has the potential to proliferate in the epithelium of upper and lower respiratory tract tissues, the digestive tract, testes, oviduct, kidneys (Cavanagh 2007) and proventriculus (Yu *et al.*, 2001; Ganapathy *et al.*, 2012). It has been shown that during virulent IBV infection, different chicken breeds may show variable disease outcomes, which could be associated with the magnitude of the immune response Poultry production faces several challenges to remain sustainable, including outbreaks of flock viral infection such as with IBV (Boroomand *et al.*, 2012). These infections cause an increase in mortality and decline in egg production. It is also possible that heavy genetic selection for fast growth rates may have made some breeds more susceptible to infections compared to others (Zekarias *et al.*, 2002). As IBV control is complicated by poor vaccine cross-protection(Jackwood and de Wit 2013), an alternative way to control infection is by selective breeding of resistant chicken lines, for which the adaptive and innate host immune responses will vary depending on the pathogen (Janeway *et al.*, 2001), the environment and host fitness. Adaptive immunity is dependent on an initial exposure followed by a specific T memory cell response. In comparison, the innate response is non-specific, acts for a broad range of pathogens and is the first line of defence from infection by utilising proteins such as pattern recognition receptors (PRRs), interferons (IFNs) and pro-inflammatory cytokines (Chhabra *et al.*, 2015a).

A number of studies have investigated the pathogenesis of several IBV variants within different chicken lines, including SPF, commercial layer and commercial broiler chickens (Ignjatovic *et al.*, 2002; Terregino *et al.*, 2008; Benyeda *et al.*, 2009; Boroomand *et al.*, 2012; Dolz *et al.*, 2012; Awad *et al.*, 2016; Khataby *et al.*, 2016). Similarly, there has been growing interest in studies that analyse the genes that can influence the course of infection

in different breeds and highlight potential genes for resistance to IBV infection (Smith *et al.*, 2015; Chhabra 2016; He *et al.*, 2016). It has been emphasised that the resistance to IBV M41 seen in chicken line N was due to host response following infection, rather than how the host prevented cell attachment and invasion (Smith *et al.*, 2015). It has been reported how each IBV variant was associated with differing tracheal and kidney innate immune responses when three strains were compared to each other *in vivo* (Chhabra *et al.*, 2018).

Several studies have demonstrated that there are differences in gene expression in fast and slow growing breeds following heat stress (Rimoldi *et al.*, 2015), *Salmonella* infection (van Hemert *et al.*, 2006), feed removal (Benyi *et al.*, 2010) or daily feed skip restriction in a specific environment (Netshipale *et al.*, 2012). Smith and others highlighted that the differences in susceptibility to IBV by two different lines (susceptible - 15I, inbred White Leghorn *versus* resistant - Line N, non-inbred Cornell) of chickens (Smith *et al.*, 2015). Other studies have been reported on the difference in immune responses in different broilers lines when infected by *Campylobacter jejuni* (Williams *et al.*, 2013; Humphrey *et al.*, 2015). Despite a significant number of IBV pathogenesis studies, there is limited information comparing immunopathogenesis of IBV in different breeds of commercial chickens. This study aimed to examine the differences between two commercial broiler breeds, fast grower Line-A *versus* slow grower Line-B, following infection by an IBV strain Q1. Furthermore, the innate immune and pro-inflammatory cytokines responses in these two lines of birds were examined following infection (Manswr et al., submitted).

5.2 Materials and Methods

5.2.1 Virus

The IBV Q1 virus strain reference number 37089/2013 was propagated in embryonated SPF eggs and titrated in TOCs to determine the relative titre (Chapter 2.2).

5.2.2 Chick, welfare and management

The fast (Line A) and slow (Line B) growing reaches live slaughter weight (2.2 Kg) at 36 and 56 days old respectively. Chicks were supplied from two different commercial hatcheries. Chicks were kept up to 28 days of age (doa) in an isolation unit (University of Liverpool) throughout the experiment. Chicks were reared on wood shaving litter with food and water supplied *ad libitum* (Chapter 2.4).

5.2.3 Experimental design

Two breeds of broiler Line-A and Line-B chickens were at a total number of 80 from each Line. From each Line chicks were allocated in two groups control and infected in total number of 40 in each group (n=40). Chicks in the infected groups were inoculated oculonasally with $10^{4.5}$ CD_{50}/bird of IBV Q1 in a volume of 100µl and chicks in the control groups were inoculated with 100µl virus-free allantoic fluid. At weekly intervals the body weight of 10 randomly picked chicks from each group were weighed and clinical signs were observed daily. Oropharyngeal (OP) and cloacal (CL) swabs were collected from ten birds at 3, 7, 9, 14, 21, 28 dpi. Trachea and kidney are scored for gross lesions after chicks humanely been killed and euthanized at 1, 3, 7, 9, 14, 21, 28 dpi. Five chicks from each group were sampled from the next tissues; trachea, kidney and proventriculus. Sections of these tissues were collected in the RNAlater (Qiagen, Crawley, UK), and stored at -20 ºC for later processing for virus load and expression of pro inflammatory cytokines and host genes mRNAs expression . In the same time, another portion of samples, kidney and trachea were also collected in 10% buffered formalin for histological examination. One portion from the proventriculus was used for virus isolation. At 1,7,14, 21, 28 dpi five birds from each group were bled, sera was separated and stored at -20 ºC for detection of IBV antibodies by ELISA.

5.2.4 Swab samples

Dry swabs were used to sample the cloaca and oropharyngeal cavity at 3, 7, 9, 14, 21, and 28 dpi for RT-PCR and qRT-PCR. After sampling, swabs from each group were pooled, dipped into 2.5ml of TOC media, and stored at -70^{o}C until required.

5.2.5 Clinical signs

Clinical signs were recorded daily according to previously published scoring system (Grgic *et al.,* 2008), (Chapter 2. 8.1).

5.2.6 Pathological changes

Five birds from each group was humanely euthanized on 1, 3, 7, 9, 14, 21 and 28 dpi and the trachea, kidney and proventriculus were removed. Gross lesions were recorded as previously described (Mahgoub *et al.,* 2010), (Chapter 2.8.2).

5.2.7 Histology

Histological changes were scored as 0 = no change, 1= mild, 2 = moderate, 3 = severe as mentioned previously (Chen and Itakura 1996; Chhabra 2016), (Chapter 2.11)

5.2.8 RNA extraction from swabs and allantoic fluid

The RNA was extracted from the OP and CL swabs or allantoic fluid (AF) using the phenol-chloroform method for RNA extraction as mentioned previously (Chomczynski and Sacchi 2006; Christo *et al.,* 2016). Total RNA was extracted from all collected tissues, extraction of viral RNA was conducted using the RNA easy kit (Qiagen) according to the manufacturer's instructions(Awad *et al.,* 2014), (Chapter 2.12).

5.2.9 Serum

Blood was collected from the brachial wing vein and placed in labelled 5 ml tubes without coagulant. Sera was collected and stored at -20 ^{o}C until needed for ELISA (Chapter 2.7.2).

5.2.10 Reverse transcription polymerase chain reaction RT-PCR

IBV RT-PCR was conducted on swabs and allantoic fluid, as previously described (Worthington *et al.,* 2008; Ganapathy *et al.,* 2015; Ball *et al.,* 2016c) that targeted a 393bp portion of the S1 gene which allowed to sequence the hypervariable region of the S1 gene (Chapter 2.15).

5.2.11 Purification for sequencing

The OP and CL swabs, and tissues that were positive for IBV by RT-PCR were sequenced for the part-S1 gene as illustrated in (Chapter 2.17). Purification of the nested PCR product was applied using shrimp alkaline phosphatase (SAP) mixed with Exonuclease (EXO) (Chapter 2.15.5).

5.2.12 IBV Quantitative reverse transcription PCR (qRT-PCR)

Quantification of the viral RNA was carried out using qRT-PCR as previously described (Jones *et al.,* 2011). All reactions were performed using the One-Step RT-PCR Qiagen kit and 40 ng of total RNA per reaction (Chapter 2.18).

5.2.13 Expression of host genes (qRT-PCR for host gene expression analysis)

After 1, 3, 7, 9, and 14 dpi the host gene expression analysis were carried out for gene and cytokine mRNA expression using qRT-PCR of cDNA for the relative expression analysis of selected genes was carried out as (Kuchipudi *et al*., 2012).Data was normalised using a relative standard curve method to 18S ribosomal RNA (18srRNA) expression and the data were presented as fold difference in gene expression of virus vs mock infected samples (Chapter 2.19).

5.2.14 Tissue samples for viral isolation and RT- PCR from the proventriculus

Sampled tissue was grinded through a sterile pestle and mortar and using a sterile sand and 100µl of TOC media. Grinding was done in a septic condition and near flame. Tissue,

suspension was stored at -70 ^{o}C until process. When the sampled tissue dilution processed it centrifuged at 3,000 g for 10m before being used (Chapter 2.10). Virus isolation was carried out from proventriculus samples taken at 7 and 9 dpi following tissue grinding.The RT-PCR positive samples were sent for commercial bi-directional sequencing (Source BioScience Ltd, Nottingham, UK), (Chapter 2.15).

5.2.15 Serology

Serum samples were analysed using a commercial IBV ELISA kit (IDEXX) according to manufacturer's instructions. Sera were diluted in a ratio of 1:500 with diluent and added to coated antigen plates (Chapter 2.20).

5.2.16 Statistical analysis

Parametric and non-parametric data were analysed using graph pad prism software 7 using one-way ANOVA followed by LSD test and Kruskal-Wallis test followed by Dunn's test respectively. Differences were considered significant at $p<0.05$ (Chapter 2.22).

5.3 Results

5.3.1 Body weight

A significant reduction ($P<0.05$) in the mean body weight was seen in the infected Line-A group at 14, 21 dpi in comparison with the control group. Body weight was lower at 7 and 28 dpi, however this was not significant (Figure 5.1A). While there was a reduction in the infected Line-B body weights at all time points, none were considered significant ($p>0.05$) (Figure 5.1B). There were significant differences ($P<0.05$) in the average body weight

between both the Line-A and Line-B controls and infected groups at 1, 21 and 28 dpi (Figure 5. 1C and Figure 5.1D).

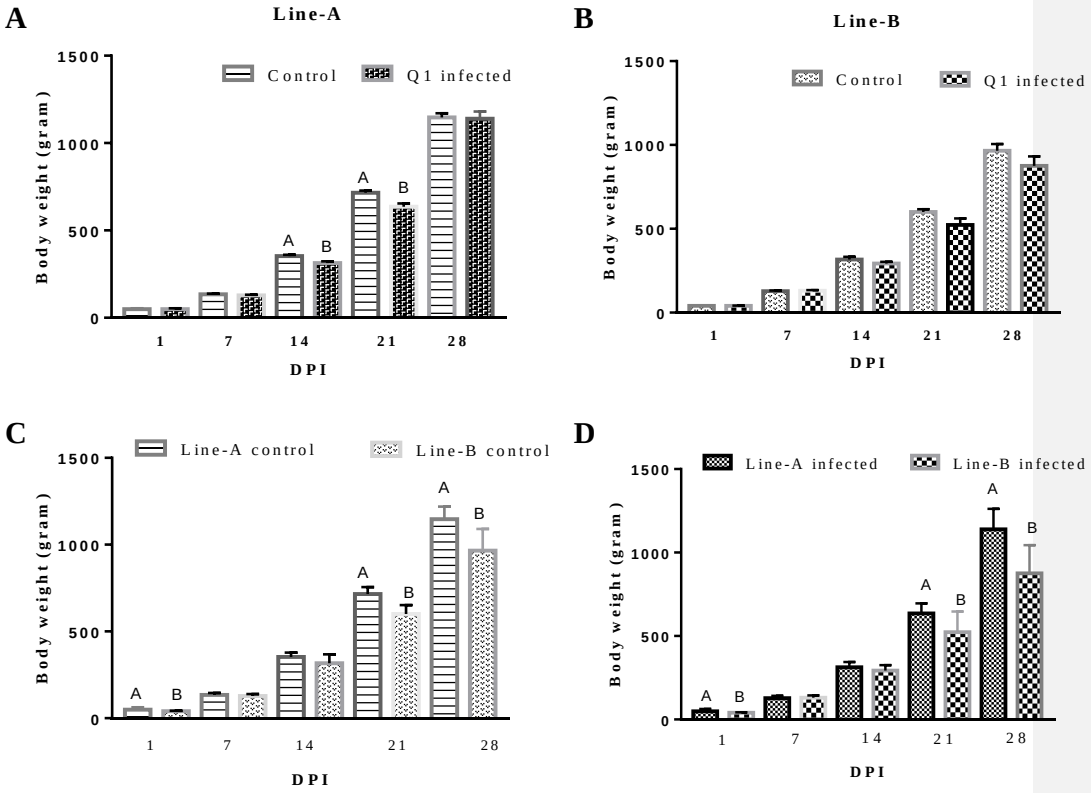

Figure 5.1. Weekly mean body weight comparisons at 1, 7, 14, 21 and 28 dpi; (A) Line-A control and infected groups, (B) Line-B control and infected groups, (C) Line-A and Line-B controls and (D) Line-A and line-B infected groups at all sampling days. Data represents the mean with error bars as standard error.

5.3.2 Clinical signs

For both lines, there were no clinical signs or mortalities in the control groups. In both infected groups, signs started at 3 dpi and lasted until 20 and 21 dpi for Line-A and Line-B infected groups respectively, with moderate signs present until 14 dpi in Line-A and 13 dpi in line-B (Figure 5.2). Signs included sneezing, tracheal râles, coughing, head shaking, nasal exudate, eye scratching and mild diarrhoea. At 5 dpi, a single mortality was recorded in the infected Line-A group.

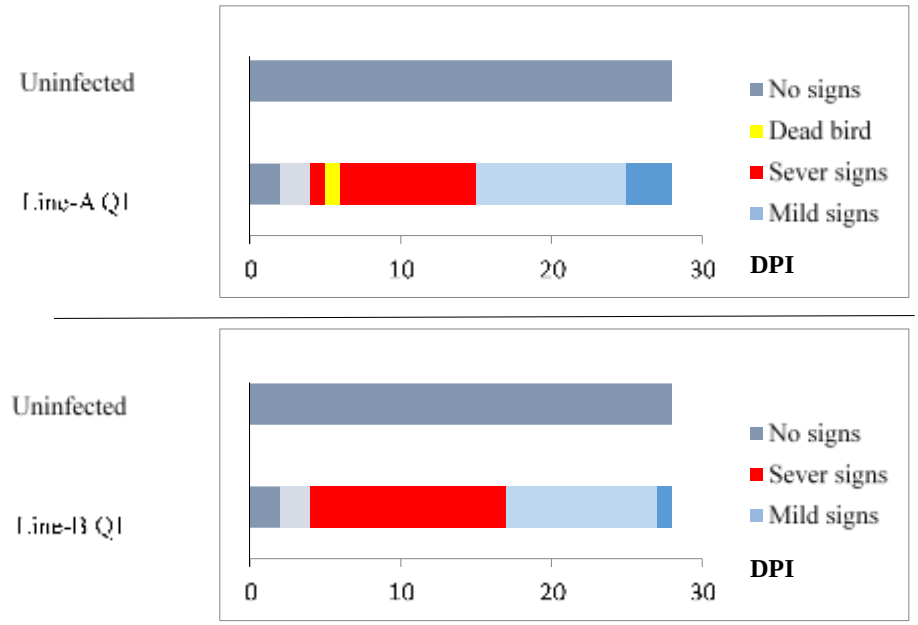

Figure 5.2. Shows the onset and duration of clinical signs and mortality within the period of the experiment (28 days) in Line-B and Line-A control and infected group.

5.3.3 Gross lesions

There were no gross lesions observed in the Line-A and Line-B control groups. In both Line-A and Line-B infected groups there was congestion, hyperaemia, and excessive

exudate in the trachea (Figure 5.4). There was fibrinous pericarditis in the infected Line-A group at 14 dpi (1 of 5 birds) and fibrinous pericarditis and fibrinous perihepatitis in the infected Line-B group at 14 dpi (1 of 5 birds). In both infected groups, kidney lesions consisting of paleness and swelling and urate deposition with lower severity lesions in Line-B infected group. The mean lesion scores show that overall the Line-A infected group produced higher average lesion scores for both trachea and kidney in comparison to the Line-B infected group (Figure 5.3A and 3B). There were no noticeable gross lesions at day 21 and 28 dpi for any groups.

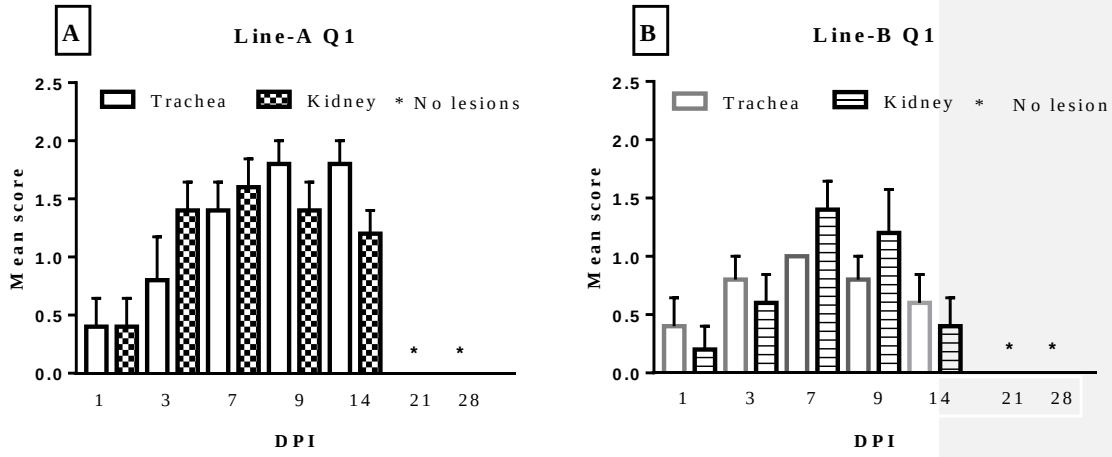

Figure 5.3. Gross lesion score for (A) infected Line-A trachea and kidney and (B) infected Line-B trachea and kidney. Birds were examined for gross lesions at 1, 3, 7, 9, 14, 21 and 28 days post infection (dpi). Data represents the mean with error bars as standard error.* = No lesions.

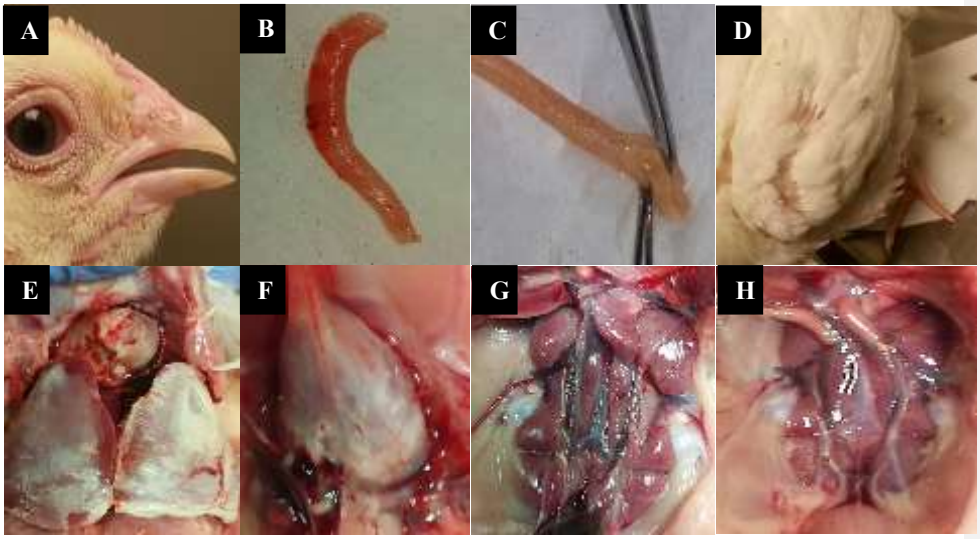

Figure 5.4. Development of the gross lesion after IBV Q1 oculo-nasal inoculation into broilers line-A and line-B Infected groups at one day old. (A). conjunctivitis and nasal exudates after gentle pressing in Line-A at 9 dpi. (B, C). Trachea revealed different degrees of congestion and exudates in Line-A Infected group and Line-B Infected group (D). Diarrhoea in Line-A Infected group at 21 dpi. (E). Fibrinous pericarditis, fibrinous per hepatitis and airsacculitis in Line-B Infected group at 14 dpi, (F). Fibrinous pericarditis in Line-A Infected group at 14 dpi. (G). Line-B Infected group, swelling kidneys with nephritis and deposition of uric acid in ureters at 14 dpi (H). Line-A Infected group, swelling kidneys with nephritis and deposition of uric acid in ureters at 14 dpi

5.3.4 Histological changes

There were no significant differences in tracheal histological changes between infected groups for any time point. Tracheal lesions of infected Line-A and Line-B groups consisted mainly of deciliation and epithelial degeneration, decreased mucous cells and heterophil infiltration (Figure 5.6C and 5.6D). Lesions started early at one dpi and continued up to 28

dpi. The highest tracheal histopathological scores were seen at 9 and 7 dpi for Line-A and Line-B respectively (Figure 5.5A).

Kidney lesions appeared at 3 dpi in both infected groups and peaked at 21 and 14 dpi in Line-A and Line-B respectively. Kidney histological changes consisted of epithelial degeneration, lymphoid infiltration and lymphoid nodules (Figure 5.6G and 5.6H). Similar to the trachea, changes continued until 28 dpi (Figure 5.5B). There were no histopathological changes seen in the proventriculus for the infected Line-B group, whilst lesions comprised mostly of glandular sinus dilation and lymphoid infiltration and scattered lymphoid follicles were seen at 7 and 9 dpi in the infected Line-A group.

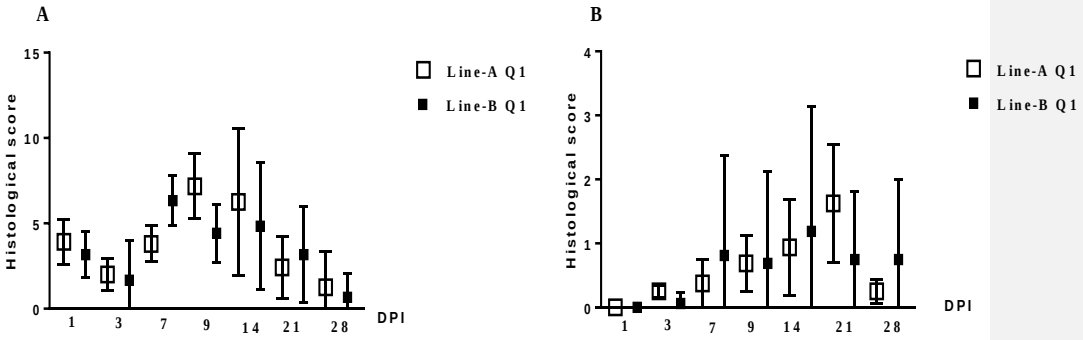

Figure 5.5. Histological score of lesions in the trachea and kidney of infected groups (A) Line-A and Line-B infected group tracheal histological score and (B), Line-A and Line-B infected groups renal histological score. Birds (n=5/group) were infected with IBV strains Q1 at a dose of $10^{4.5}$ CD_{50}/bird and post mortem tissue was processed for histopathological examination at 1, 3, 7, 9, 14 , 21 and 28 days post infection (dpi). Data represents the mean with error bars as standard error.

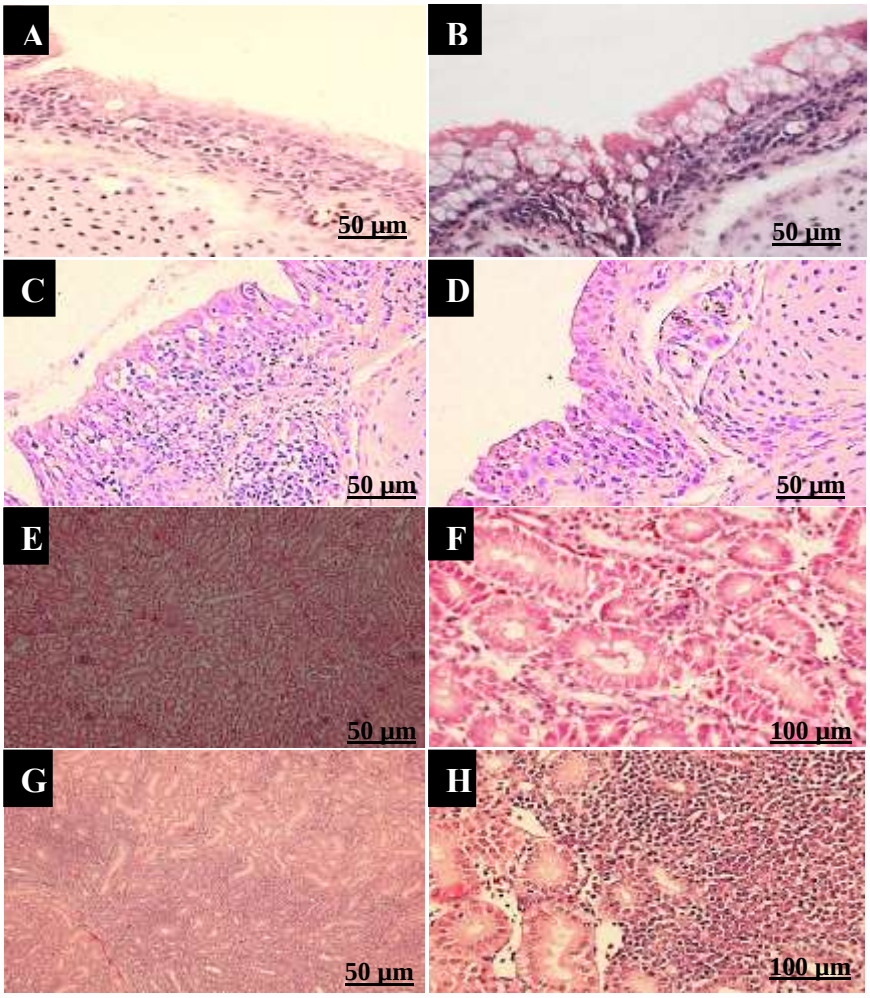

Figure 5.6. Histological findings in trachea and kidney samples, using H&E stain.(A) Uninfected trachea from Line-A group showing cilia and goblet cells (B) Uninfected control Line-B group showing cilia and goblet cells. (C) Trachea from Line-A infected group at 9 dpi, extensive epithelial deciliation. (D) Trachea from Line-B infected group 9 dpi with lymphocyte and heterophil infiltration. (E) Kidney from Line-A uninfected control group (F) Kidney from Line-A uninfected control group (G) Kidney Line-A infected group, tubular degeneration. (H) Kidney from Line-A infected group moderate lymphocyte and mild heterophil interstitial infiltration in kidney at 9 dpi.

5.3.4 Detection of Q1

5.3.4.1 Pooled oropharyngeal (OP) and cloacal (CL) swabs RT PCR:

In the Line-A infected group, all collected OP and CL swabs were positive for IBV by RT-PCR. While, in the Line-B infected group, both swab sites were positive until 14 dpi. Amino acid (AA) changes were present early, in the Line-A infected group, with OP sequence changes at 3 dpi, which consist of 9 AA. In addition, Line-A 3 dpi OP swabs, there were 3 AA deletions consisting of asparagine (N), phenyl alanine (F) and asparagine (N) respectively at position 308-310 at AA level in the S1 gene. At 9 dpi, there was 1 AA change in Line-A OP swab sequence from valine (V) to lysine (K). At 14 dpi, there were 2 AA changes, one threonine (T) to serine (S) and the other one being valine (V) to lysine (K). At 21 dpi, there were 7 AA changes which were not present at 28 dpi. Noticeably, no AA changes were found in the Line-B infected group OP swab sequences at any sampling days.

In the CL swab sequences, AA changes were present at 7 dpi, which consisted of 27 AA in the Line-A infected group. Whilst, in the Line-B infected group, there were 4 AA changes from CL swab sequences at 7 dpi, these being serine(S) to threonine (T); phenylalanine (F) to isoleucine (I); arginine(R) to glutamine (Q) and leucine (L) to lysine (K) , respectively. At 28 dpi, there was one AA change in the Line-A CL swab sequence from serine(S) to threonine (T)

(Table 5.1).

5.3.4.2 qRT PCR from swabs:

All infected OP and CL samples were IBV positive, with the exception of 28 dpi in the OP swabs (both lines) and 28 dpi in the CL swabs (Line-B only). On average, the viral load from OP swabs was lower than from CL swabs in both infected groups, and both the OP

and CL swabs from the infected Line-A group had a higher viral load compared to swabs from Line-B (Figure 5.7).

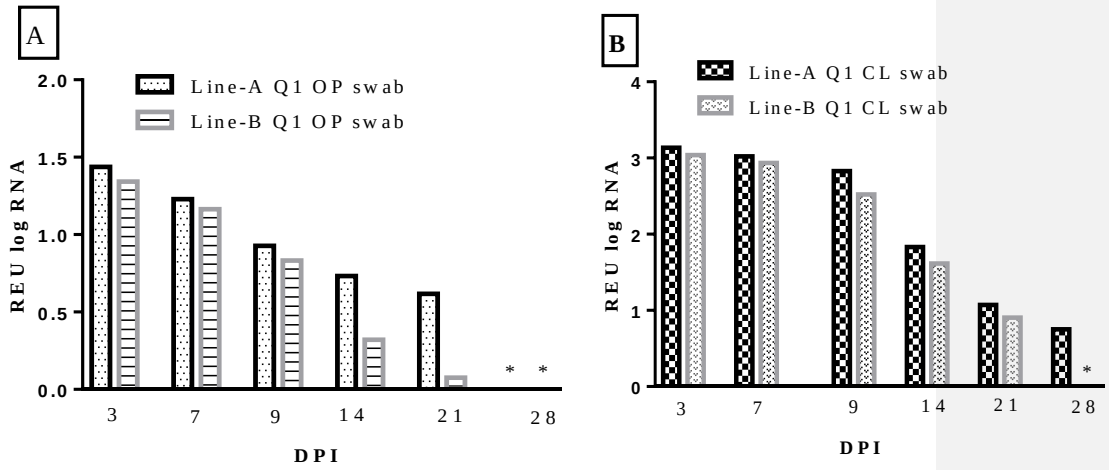

Figure 5.7. Quantification of viral RNA from OP and CL swabs of infected groups and, expressed as a log relative equivalent units (REU) of RNA in the OP and CL swabs of infected Line-A group and Line-B. Birds (n=10/group) were infected with IBV strain Q1 at a dose of $10^{4.5}$ CD_{50}/bird and analysed for quantification of viral RNA at 3, 7, 9, 14, 21 and 28 days post infection (dpi). Data represents the mean from 10 swabs.* Not detected.

Table 5.1. Sequence alignment of the S1 glycoprotein of IBV Q1 strain from OP and CL swabs at 3, 7, 9, 14, 21 and 28 dpi. Dots indicate identical amino acid to the original inoculum (first row of aligned sequences). Letters denote change in the amino acid. Dashes denote deleted amino acids.

OP swabs

```
Ino:TTLVLTNFTFSNVSNAPPNTGGVHSIVLHQTQTAQSGYYNFNFSFLSSFRYVESDFMYGSYHPKCSFRLETINNGLWFNSLSVSLGYGPLQGGC
A 3 ...M.....................................___..........A.........S..K.Q......L...L...R....P...
B 3 ..........................................................................................
A 7 ..........................................................................................
B 7 ..........................................................................................
A 9 ...K......................................................................................
B 9 ..........................................................................................
A 14S..K.....................................................................................
B 14.........................................................................................
A 21...R.....................................................G........K..G...F.G...N.......
A 28.........................................................................................
```

CL swabs

```
Ino:TTLVLTNFTFSNVSNAPPNTGGVHSIVLHQTQTAQSGYYNFNFSFLSSFRYVESDFMYGSYHPKCSFRLETINNGLWFNSLSVSLGYGPLQGGC
A 3 ..........................................................................................
B 3 ..........................................................................................
A 7 ...M.....................................FH.S....I.LSNI.AY..P..K....S.IKQ.S.EID...FPW..P.T.....V.
B 7 ..........................................................TIKQ..............................
A 9 ..........................................................................................
B 9 ..........................................................................................
A 14.........................................................................................
B 14.........................................................................................
A 21.........................................................................................
A 28.............................................T............................................
```

5.3.4.3 qRT PCR of tissues

At 7 dpi, the tracheal tissue of the infected Line-A group had a significantly ($P<0.05$) higher viral load when compared to Line-B (Figure 5.8A). At 7-9 dpi, there was a significantly ($P<0.05$) higher differences in the viral load of the kidney of infected Line-A group when compared with the Line-B infected group (Figure 5.8B). The IBV viral load was detected in the proventriculus between 1-9 dpi in Line-A and 3-9 dpi in Line-B. Line-A showed a significantly ($P<0.05$) higher viral load compare to Line-B at 7 - 9 dpi (Figure 5.8C). No IBV was detected at 21 and 28 dpi for any tissues.

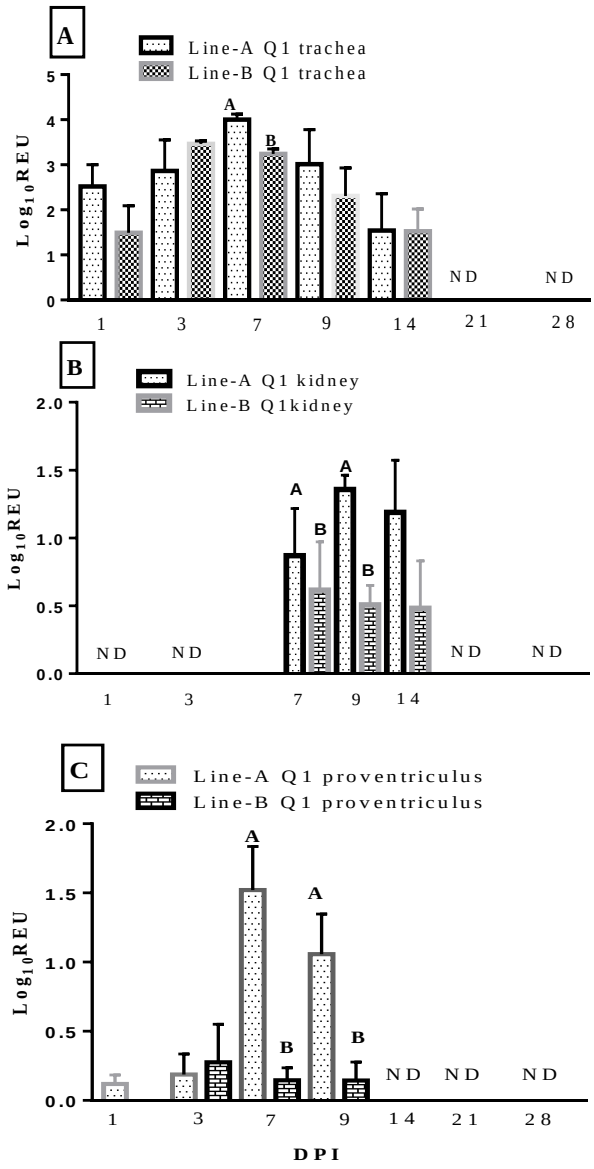

Figure 5.8 .Quantification of viral RNA in the (A) trachea, (B) and (C) proventriculus form infected groups of Line-A and Line-B, expressed as a log relative equivalent units (REU) of RNA in the Tissues of infected Line-A and Line-B groups. Data represents the mean with error bars as standard error.

5.3.4.4 Isolation of IBV Q1 from proventriculus

The re-isolation of Q1 was attempted from samples of proventriculus at 7 and 9 dpi. Isolation was successful for both Line-A and Line-B and upon partial S1 sequencing; the isolates had 99 % amino acid similarity to the initial inoculum.

5.3.4.5 IBV ELISA antibodies

The mean maternal derived antibody (MDA) was 1720 and 3340 in Line-A and Line-B respectively. The mean antibody (Ab) titre in the Line-A infected group was 100 and 216 at 21 and 28 dpi respectively (Figure 5.9A). While in the line -B infected group, it was 290 and 510 respectively. In the Line-A infected group, there were positive ELISA antibodies detected by 21 and 28 dpi in 11% and 25% of birds respectively. In the Line-B infected group, the percentage of birds with positive ELISA antibodies at 21 and 28 dpi were 37.5% and 62.5% respectively (Figure 5.9 B).

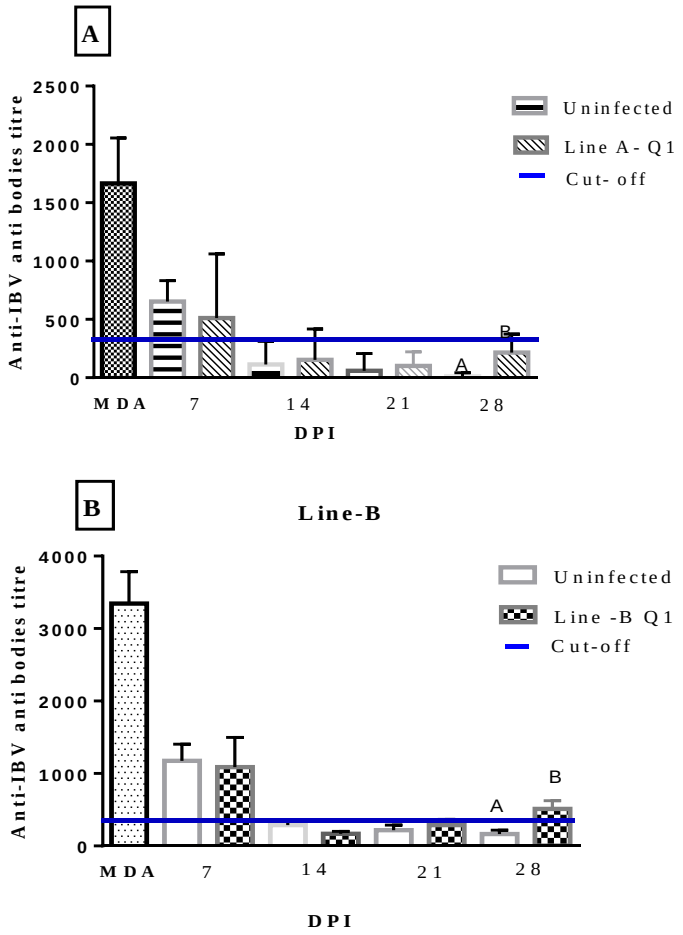

Figure 5.9. Mean infectious bronchitis virus (IBV) antibody titres of the control and infected groups (n=10/group) at 21 and 28 days-old. Data are expressed as mean values ± SEM. The ELISA cut-off is 396.

5.3.5 Host gene expression analysis

5.3.5.1 Relative IFN-α and IFN-β mRNA expression

5.3.5.1.1 Trachea

In the Line-A infected group, there was significant up-regulation ($P<0.05$) in the mRNA expression of IFN-α between 1-14 dpi when compared to the control group. Expression peaked at 1 and 3 dpi, then gradually declined until 14 dpi (Figure 5.10A). Following the same trend as Line-A, there was an up-regulation in the infected Line-B group at all sampling days. Data for IFN-β expression in the infected Line-A group revealed significant up-regulation ($P<0.05$) at all sampling days, except at 14 dpi when compared with control group (Figure 5.10B). Whereas data from Line-B showed significant ($P<0.05$) up-regulation in all sampling days, with the peak almost at 100 times fold change at 3 dpi.

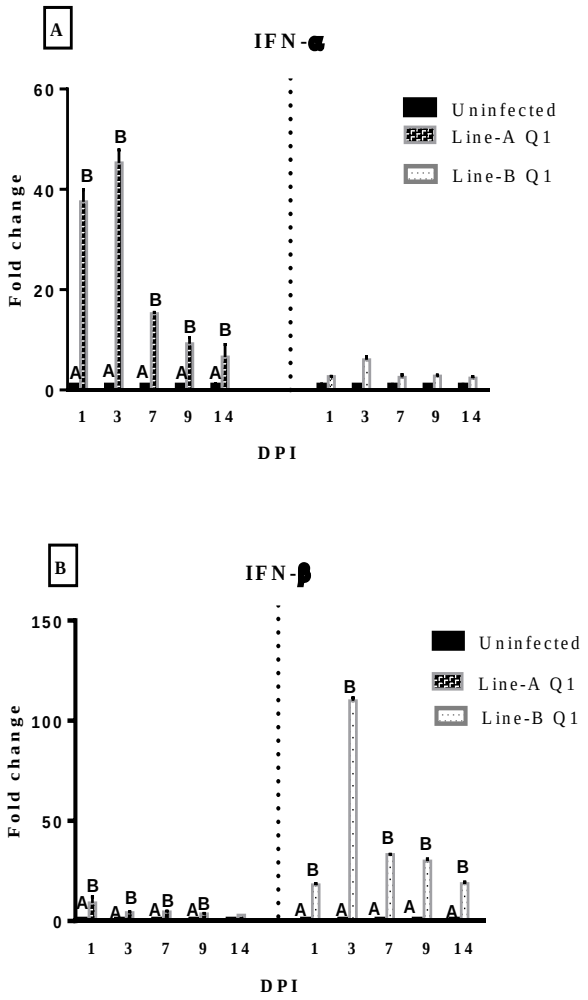

Figure 5.10. Relative type I interferon (IFNs) mRNA expression in Line-A and Line-B control and infected groups: (A) IFN-α expression in the trachea (B) IFN- β in the trachea. Significant differences between the groups were detected by t-test, different letters indicates significant differences ($P<0.05$).

5.3.5.1.2 Kidney

In the Line-A infected group, there was significant up-regulation ($P<0.05$) of IFN-α mRNA expression at 1 dpi when compared to the control group (Figure 5.11A). There was down regulation in the other sampling days. In the Line-B infected group, there was up-regulation in all sampling days in the mRNA expression of IFN-α without any significant when compared to control group. The peak point was at 7 dpi (Figure 5.11A). In general, the Line-B infected group exhibited higher up-regulation from 3-14 dpi when compared to the Line-A infected group.

In the Line-A infected group, there was significant up-regulation ($P<0.05$) in the mRNA expression of IFN-β at 1-3 dpi. There was up-regulation in the other sampling days when compared to the control uninfected group (Figure 5.11B).

In the Line-B infected group, there was slight up-regulation in the mRNA expression of IFN-β in all sampling days in comparison to the control uninfected group (Figure 5.11B). The Line-A infected group showed higher up-regulation when compared to the Line-B infected group.

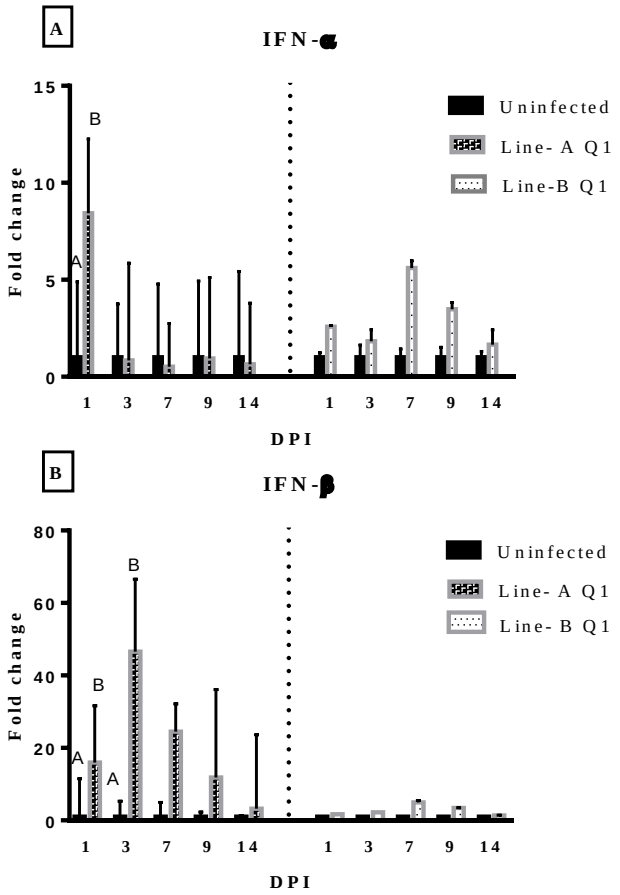

Figure 5.11. Relative type I interferon (IFNs) mRNA expression in Line-A and Line-B control and infected groups: (A) IFN-α expression in the kidney (B) IFN- β in the kidney. Significant differences between the groups were detected by t-test, different letters indicates significant differences ($P<0.05$).

5.3.5.2 Expression of mRNA of TLR3 and MDA5 in the trachea and kidney

5.3.5.2.1 Trachea

There was significant ($P<0.05$) up-regulation of TLR3 expression for Line-A between 1-7 dpi (Figure 5.12A). The same pattern was seen for Line-B, except up-regulation continued until 14 dpi (Figure 5.12). Generally, TLR3 was up-regulated to a greater extent in Line-A

compared to Line-B. There was a significant up-regulation of MDA5 for Line-A at 1 dpi, whereas Line-B was significantly down-regulated (Figure 5.12B). No further changes were seen in the subsequent sampling times (Figure 5.12B).

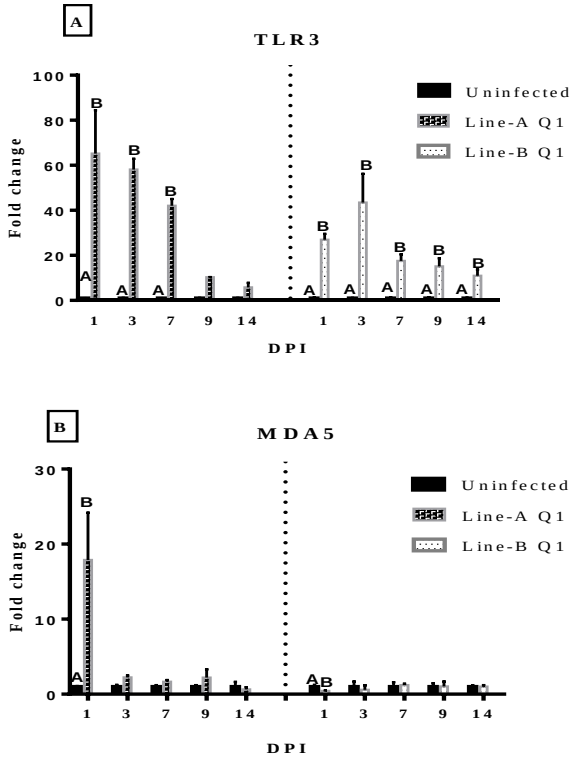

Figure 5.12. Transcriptional regulation of innate viral sensing molecules in the trachea (A) TLR3 and (B) MDA5 in the trachea of Line-A and Line-B control and infected groups. Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected T test, different letters indicate significant differences ($P<0.05$).

5.3.5.2.2 Kidney

Higher up-regulation of TLR3 mRNA expression was seen in the Line-A infected group between 1-9 dpi when compared to the control uninfected group (Figure 5.13A). In the Line-B infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of TLR3 between 1-14 dpi when compared to control uninfected group (Figure 5.13A). In the Line-A infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of MDA5 between 7-9. Up-regula regulation was seen in the remaining sampling days when compared to the control uninfected group (Figure 5.13B). The peak point was at 9 dpi in the Line-A infected group. In the Line-B infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of MDA5 between 7-9 dpi (Figure 5.13B). The extent of MDA5 was higher in the Line-A infected group, for all sampling day and roughly up to a 6 fold increase.

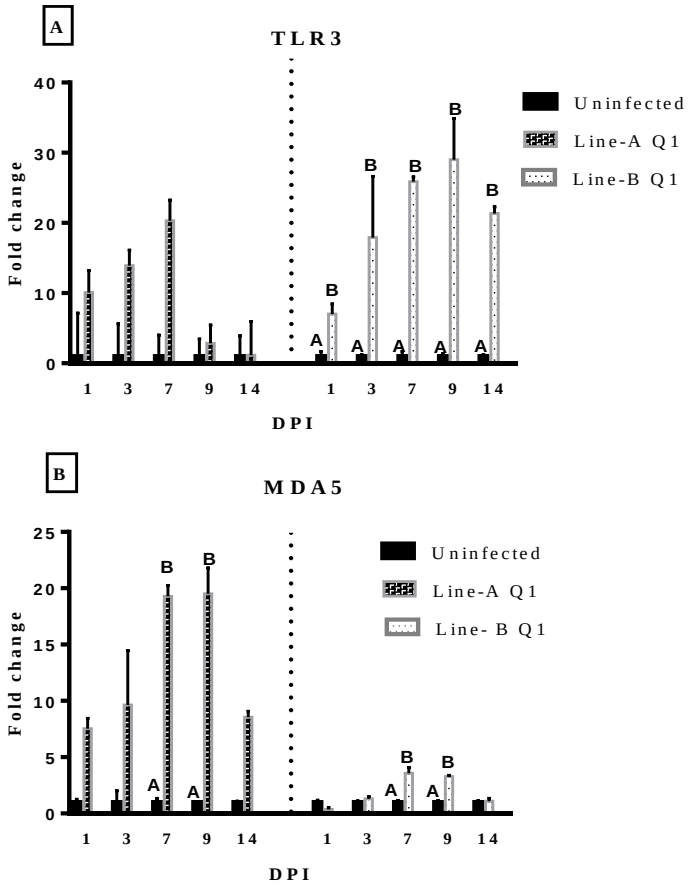

Figure 5.13. Transcriptional regulation of innate viral sensing molecules in the kidney (A) TLR3 and (B) MDA5 in the kidney in Line-A and Line-B control an infected groups. Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected T test, different letters indicate significant differences ($P<0.05$).

5.3.5.3 Transcription profile of pro-Inflammatory cytokines in the trachea and kidney

5.3.5.3.1 Trachea

For Line-A, there was significant ($P<0.05$) up-regulation of IL-1β mRNA expression between 1-14 dpi, which peaked at 1 dpi and steadily declined until 14 dpi (Figure 5.14A). A similar pattern was seen for Line-B, however only 1 dpi was significant and expression returned to the base level by 9 dpi (Figure 5.14A).

There was significant up-regulation of IL-6 expression in Line-A between 3-9 dpi, which peaked at 9 dpi (Figure 5.14B). Interestingly, there was initial significant down-regulation for Line-B at 1 dpi, which was then up-regulated from 3-14 dpi (Figure 5.14B).

Down-regulation was seen for LITAF expression, in Line-A, for all sampling points (Figure 5.14C). In Line-B infected group, there was down-regulation of mRNA expression of LITAF at 1-3 dpi which gradually increased until 14 dpi when compared to control uninfected group (Figure 5.14C).

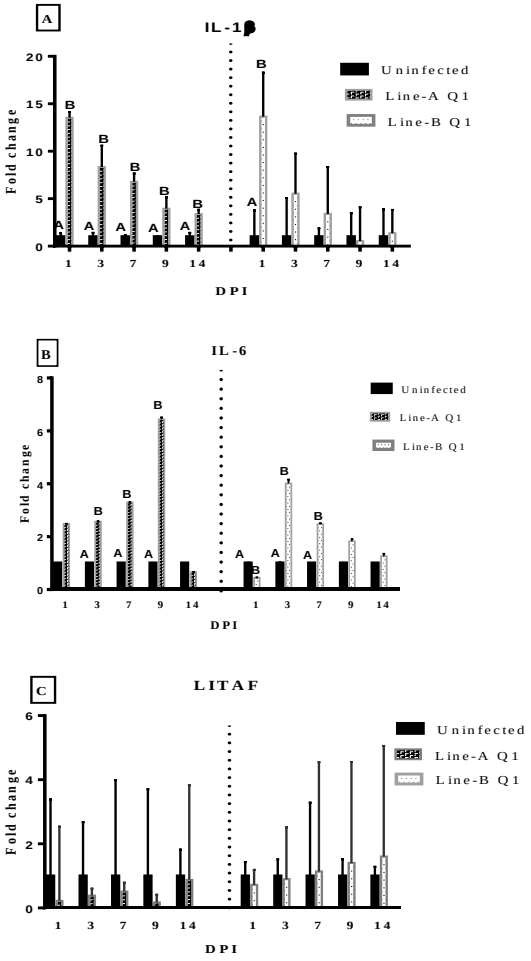

Figure 5.14. Transcription profile of pro-Inflammatory cytokines in the trachea (A) IL-1β and (B) IL- 6 and (C) LITAF in Line-A and Line-B trachea from control and infected groups. Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18s rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected t-test, different letters indicates significant differences ($P<0.05$).

5.3.5.3.2 Kidney

In the Line-A infected group, there was up-regulation in the mRNA expression of IL-1β between 1-7 dpi. In the Line-A infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of IL-1β between 9-14 dpi when compared to control uninfected group. The magnitude of up regulation in line-A was nearly 20 times more than the control uninfected group at 9 dpi when it was at the peak point (Figure 5.15A). Unlike Line-A, in the Line-B infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of IL-1β between 1-14 dpi which peaked at 9 dpi to up about 80 fold (Figure 5.15A).

In the Line-A infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of IL-6 between 7-9 dpi (Figure 5.15B). The peak point was at 9 dpi at an approximately a 6 fold increase compared to the control uninfected group. In the Line-B infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of IL-6 between 3-14 dpi (Figure 5.15B). The peak point was at 9 dpi to approximately 20 times more than the control uninfected group.

In the Line-A infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of LITAF between 7- 9 dpi when compared to the control uninfected group. The peak point was at 9 dpi (Figure 5.15C). In the Line-B infected group, there was significant ($P<0.05$) up-regulation in the mRNA expression of LITAF between 1-14 dpi when compared to control uninfected group (Figure 5.15C).

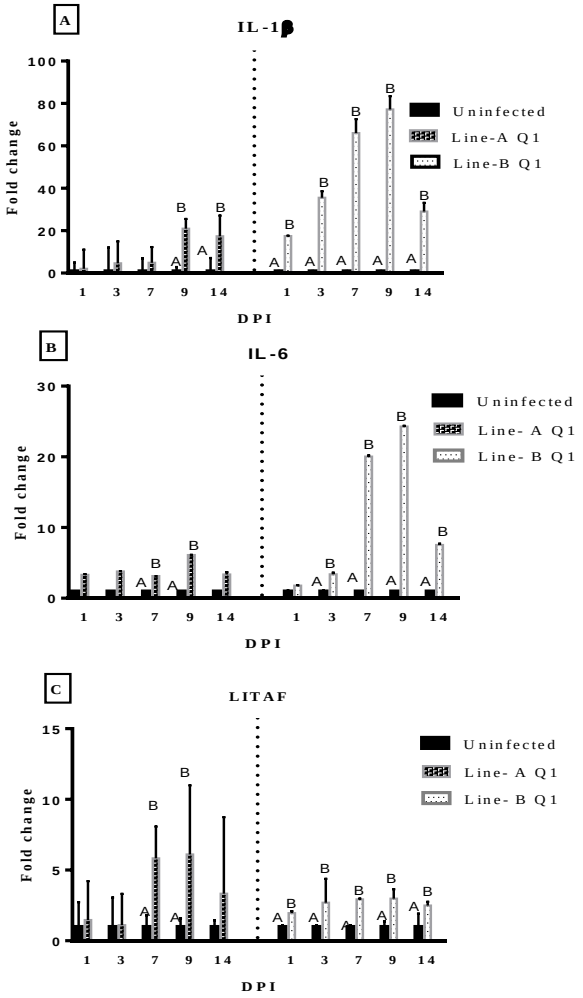

Figure 5.15. Transcription profile of pro-inflammatory cytokines in the kidney (A) IL-1β and (B) IL- 6 and (C) LITAF in Line-A and Line-B kidneys from control and infected groups. Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected t-test different letters indicates significant differences ($P<0.05$).

5.4 Discussion

In this study, the pathogenicity and host gene expression responses to Q1 infection, were studied in two broiler lines with different growth rates. Though Q1 has been reported in many countries (Ababneh *et al.,* 2012; Ganapathy *et al.,* 2015; Sesti *et al.,* 2014), few reports have been published on the pathogenicity and immune responses of this important variant, particularly in commercial broiler chicks with IBV-maternal antibody . In recent years, it has been reported that slow growing broiler chickens may be more resistant to common poultry pathogens compared to fast growing birds (van Hemert *et al.,* 2006; Williams *et al.,* 2013; Humphrey *et al.,* 2015). To further investigate the differences, we studied two breeds of commercial broilers, one with fast growth (Line-A) and another with slower growth (Line-B) to assess the impact of Q1 infection and evaluate the innate and cytokines responses in the trachea and kidney.

Weekly live body weight (LBW) measurements of chicks confirmed that Line-B chicks were slow growing in comparison to Line-A. Both infected and non-infected chicks had a lower LBW at 21 and 28 dpi compared to Line-A. However, it appears that Q1-infection of Line-B birds had no significant impact on weight gain. In contrast, Q1 infection caused a reduction in the LBW of Line-A at 14 and 21 dpi. Previous work, examining the effect of QX and Q1 infection, also noted a reduction in body weight for broiler and SPF chicks (Mahgoub *et al.,* 2010; Ganapathy *et al.,* 2012). The loss of LBW for infected Line-A birds is likely associated with the greater susceptibility of this breed IBV induced disease.

The clinical signs, gross lesions and histopathology findings showed that both bird lines are susceptible to Q1 infection, however the clinical sign, severity and duration of lesions was consistently greater in Line-A than Line-B. Respiratory signs and mortality have been previously associated with Q1 infection(Yu *et al.,* 2001), however, in this study a much lower mortality rate was found. In addition, a higher mortality rate was reported for

IS/885/06 and IS/1494/06 (Awad *et al.*, 2014; Awad *et al.*, 2016). The severity of clinical signs caused by IBV vary due to many factors related to the virus, host and environment (Jackwood and de Wit 2013). The tracheal and kidney lesions reported here are similar to those reported previously in other IBV strains such as IS/885/00 and 793B (Boroomand *et al.*, 2012; Awad *et al.*, 2016). Histopathologic changes were much more severe in the trachea and kidney from Line-A compared to Line-B. The histopathologic changes in the proventriculus seen in the Line-A infected group were similar to those reported previously in commercial broiler farms with Q1 infection (Toffan *et al.*, 2013), whereas no lesions were found in the Line-B birds proventriculus. It appears that due to genetic characteristics of Line-A, it is significantly more susceptible to Q1 infection and subsequently showed a greater severity of gross and microscopic lesions compared to Line-B.

In this study, it appears that the replication of Q1 in the Line A is greater than in the Line B. This was reflected by higher levels of viral load in the swabs and tissues in the Line A compared to Line B. In addition, in the Line A, the Q1 was continuously detected up to 28 compared to 14 dpi in the Line B. These differences may be due to variations in the levels of IBV MDA (Darbyshire and Peters 1985; Terregino *et al.*, 2008; Awad *et al.*, 2016) or genetic make-up of the birds, which may have affected virus infection and clearance.

In this experiment, Line-A infected group OP swab sequences shown amino acids changes at 3, 9, 14 and 21 dpi. No changes occurred in Line-B OP swabs sequences at any of the sampling days. Such amino acid changes lead to alterations in the S1 antigen, which could allow evasion of host immune responses (Promkuntod *et al.*, 2014), since the S1 gene contains epitopes targeted by neutralizing antibodies and inducing immunity (Cavanagh 2005). The findings in this study show a high number of AA changes in the Line-A group infecting Q1 virus that may have promoted better survival in the respiratory epithelium of the chicks. As this appears to be first study to highlight such differences between breeds of

chickens, further studies would be helpful to understand the molecular evolution of virulent or vaccine strains of IBV in different breeds of chickens.

In term of humoral immune responses, results indicate that maternal antibody levels to IBV followed a similar pattern of decline in both lines of chicks, with re-emergence of active humoral antibody levels (above the positive ELISA cut-off level) started at 21 dpi in Line-B but not Line-A . The percentage of birds with IBV positive titres was higher in Line-B compared to the Line-A, reflecting a greater ability of the slow growing chicks in providing efficient immunological response to the Q1 infection. An experiment carried by Cook and colleagues (1990) indicated that IBV-resistant breed line C showed an earlier and higher degree of humoral antibody titres to IBV M41 compared to line 151 (Cook *et al.*, 1990).

Previous studies have reported conflicting data regarding host gene expression in the trachea and kidney following IBV infection (Kameka *et al.*, 2014; Chen *et al.*, 2015) and this could be related to the strain of IBV used, the breed of birds, the route or dose of inoculation, or the environment where the chicks were kept (Gough and Alexander 1979; Cook *et al.*, 1990; Caron 2010; Okino *et al.*, 2017). And most probably, a combination of the previous factors.

In the current study, Line-A birds presented with a significant up-regulation of IFN-α at 1-14 dpi, similar to previous work using SPF birds (He *et al.*, 2016). No changes were seen for IFN- α in Line-B, which may be connected to the fact that these genes can be switched off through inhibition of host protein synthesis by accessory protein 5b of IBV (Kint *et al.*, 2016). Up-regulation of IFN-α in Line-A appears to be associated with increase in the severity of clinical signs, viral load and lesions. In both lines, there was significant up-regulation of IFN-β at 1-9 dpi that continued up to 14 dpi in Line-B, a magnitude of

increase in the Line-B is substantially higher than Line-A. However, the reason for the higher levels in Line-B is unknown, it appears to be associated with more rapid virus clearance.

The recognition of pathogen-associated molecular patterns is developed by TLR3 through sensing double-stranded (ds) RNA, and then mediated immune responses, which have central and key component in host resistance (Ruan *et al.,* 2015). There was significant up-regulation of TLR3 up to 7 dpi for Line-A, however Line-B continued until 14 dpi. Okino *et al.,* (2017b) working with Brazilian A and B strains, reported a significant up-regulation of TLR3 at the end of their experiment (8 dpi). It appears that greater expression of TLR3 over a shorter period might be associated with disease severity and IBV RNA load as seen in the Line-A.

Chicken MDA5 recognise long-duplex RNA in infected cell cytoplasm (Wu *et al.,* 2013). At 1 dpi, tracheal mRNA of MDA5 in Line-A chicks had a significantly higher expression in contrast to the down regulation occurring in the Line-B. Similar to previous publications, this finding confirms that MDA5 mRNA can be detected less than 3 days following IBV infection (He *et al.,* 2016; Okino *et al.,* 2017). The higher MDA5 expression at 1 dpi could be implicated in the greater infection severity in Line-A. For Line-B, the value was down-regulated, reflecting an association between MDA5 and disease as well as viral load, as these values were much lower compared to the Line-A. It is apparent that that different breeds respond differently to the same causative agent.

There was significant up-regulation of IL1-β in Line-A at 1-14 dpi, similar to previous work (Jang *et al.,* 2013; Okino *et al.,* 2014). A different response was witnessed for Line-B, which saw only significant up-regulation at 1 dpi. Such results further suggest the participation of these cytokines in forming lesions in the trachea of Line-A (Okino *et al.,*

2014), as the duration and extent (3-9 dpi, up to 6 fold higher change) of expression of mRNA IL-6 was greater than Line-B. Such a result is consistent with gross and histological changes accompanied with higher viral load seen in Line-A. Expression of cytokines IL1-β and IL-6 could be a consequence of a secondary response to infection, not as a direct response to viral infection (Jang *et al.*, 2013). IL-6 has previously been associated with tissue damage, the extent of which depends on the IBV strain, as lesions and the pro-inflammatory response in the host could vary to a certain degree (Jang *et al.*, 2013; Okino *et al.*, 2014; Chhabra *et al*., 2015a).

In the kidney, there was significant up-regulation of IFN-α and IFN-β at 1 and 1-3 dpi respectively in Line-A. Type I IFNs have been shown to play a significant role in bird survival during viral infection of different chicken breeds to those examined in the current study (Li *et al.*, 2006). In addition, it has been demonstrated that all types of IFNs (I, II, III) are up regulated in both IB-vaccinated and non-vaccinated birds following IBV challenge (Yang *et al.*, 2018). The early overexpression of type I interferon in Line-As infected kidney, could be as a result of greater virus proliferation since the IFNs are induced directly in infected tissues in Line-A breed.

There was significant up-regulation of TLR3 at 1-14 dpi in Line-B. It has been reported that IBV induced TLR3 at 3 dpi, when *in vivo* IBVs QX, M41, IS/885/00 and ck/CH/LDL/091022 were tested in using SPF chicks (Smith *et al.*, 2015; Chhabra 2016). The continuous detection of TLR3 reflects that the expression of TLR3 could be variable in different chicken breeds, providing divergent resistance (Ruan *et al.*, 2015). For the MDA5, though up-regulation was noticed in both infected groups, it was 20 times higher in the Line-A, where more severe disease and greater viral load was found. It appears that MDA5 could be considered as an indicator for IBV disease severity, with higher values being positively associated with disease severity and viral load, and *vice-versa*.

Kidney innate immune responses have been induced in the early stage of infection after inoculation of pathogenic M41 in SPF chicks (He *et al.*, 2016). In the current study, the pro-inflammatory cytokines in the Line-B infected group started early in the kidney at 1 dpi, which continued up to 14 dpi. These differences in the kinetics could be related to the resistance in Line-B in which a more rapid inflammatory response occurred and to a greater extent that limited viral replication and prevented lesion development in the kidney. Nevertheless, the inflammatory responses in Line-A was found until 7 dpi. Similar to Line-B, an early pro-inflammatory cytokine response was seen in a resistant line (BrL) but not in a susceptible line (White Leghorn line), accompanying infection with infectious bursal disease virus (Ruby *et al.*, 2006).

This study has demonstrated that IBV Q1 is pathogenic to both slow and fast growing IBV-maternal antibody positive broiler chicks. The slow growing chicks showed earlier clearance of the virus, less severe gross and histopathological lesions, a higher percentage of birds that seroconverted with a greater titre of IBV antibodies and resisted body weight losses compared to the fast growing chicks. Innate immune responses broadly demonstrated that Line-B showed longer expression of host genes such as TLR3 in both trachea and kidney compared to Line-A, demonstrated by resilient responses in the slow growing chicks. Further studies on the differential immune responses to IBV strains by different types (broiler, layer, breeder, slow or fast growing) of chicks in relation to disease and losses could be useful for better prevention strategies against classical and variant IBVs.

Chapter 6: Pathogenesis assessment of vaccine or virulent infectious bronchitis viruses in chick embryo tracheal organ cultures

Abstract

Much uncertainty still exists regarding the relationship between *in vitro* pathogenicity (or virulence) and the genetic characteristics of different infectious bronchitis virus (IBV) strains. Three virulent (Massachusetts, 793B, and QX) and eight vaccine strains of IBV were inoculated in tracheal organ cultures (TOCs) to evaluate *in vitro* pathogenicity on ciliated epithelia of trachea. At 24 and 72 hours post infection (hpi), TOC media and tracheal rings were collected, and assessed for virus load (by RT-PCR and qRT-PCR), innate immune response and total apoptotic cell differences. Evident differences in the virulence were noted between strains within the same serotype, and certain vaccines resulted in cilia degradation comparable with the virulent strain. The average cilia motility readings in Mass group, Mass1 and VirMass reached complete ciliostasis at 96 and 72 hpi respectively. Whereas, in the 793B and QX groups complete ciliostasis was reached for all strains by 120 hpi. The qRT-PCR analysis revealed decreased viral presence in the media at 24 and 72 hpi. Differences were found between the total apoptotic cell counts in the tracheal rings among virulent and vaccine strains. Downregulation in mRNA expression of IFN-α at 24 and 72 hpi occurred in all virulent and vaccine infected TOCs. At 24 hpi, there was up-regulation in IFN-β which was down regulated by 72 hpi in virulent infected TOCs. At 24 and 72 hpi, there was up-regulation in the mRNA expression of TLR3 in all vaccine and variant strains. An up-regulation of MDA5 was seen at 24 hpi in Mass serotype strains, Vri793B, 793B1 and QX serotypes. This study demonstrates the successful use of the *in vitro* TOC model for distinguishing differences in virulence and replication rate among classical and variant strains of IBVs.

6.1 Introduction

Infectious bronchitis virus (IBV) variants are appearing regularly in large commercial chicken flocks; these variants often completely or partially escape the immune responses provided by currently available commercial live IBV classical and variant vaccines (Jackwood 2012). Vaccination is the primary method for controlling IBV infection. However, there is an emergence of variant IBV strains in commercial farms for which the current vaccines are unable to cross-protection (Bru *et al.*, 2016). Vaccines, produced from endemic IBV strains, have improved flock health against commonly circulating virulent strains. The first vaccine type produced, the Massachusetts (H120) serotype has been used globally as it was the only one available for many years (Jordan 2017). With the increasing emergence of new IBVs variants around the world, there are few available vaccines that are effective against IBV strains such as QX (Cook *et al.*, 2001; Jackwood 2012).

To reduce time, costs, and for humane purposes, an alternative method from using live experimental birds have been sought for experimentation. There are recommendations from national animal experimentation regulatory authorities to increase the use of *in vitro* biological methods such as Tracheal organ cultures (TOCs)(Cardoso *et al.*, 2005), rather than *in vivo* work. The advantages of TOCs are that they are simple to prepare and can be used to study IBV infection for example for antigenic relationships, genetic mutations and the prediction of tissue tropism (Raj and Jones 1996b; Wang *et al.*, 2012; Ball *et al.*, 2016b). Also, a several TOC rings can be prepared from a single trachea of an embryonated chick foetus, and these can be used for testing of a number of virus isolates. Variations between the groups can be kept minimum as the rings come from the same birds or group of birds.

Strain variations may be related to viral replication mechanics or as a consequence of the alteration of signalling pathways, such as cell stress and host innate immune response, to

facilitate viral pathogenesis (Zhong *et al.,* 2012). Several viruses, such as IBV, tend to use two central common strategies to infect and invade host cells which ultimately initiate apoptosis and influence the host cell cycle (Li *et al.,* 2007). In the same way, during virus infection, apoptosis can be considered an innate cellular response to limit viral propagation, however, it might also facilitate virus dissemination(Alcami and Koszinowski 2000). Recently, Chhabra et al. outlined an association between apoptosis and an increase in specific mRNA gene expression which also indicated the IBV tissue tropism. The up-regulation of Toll-like receptor 3 (TLR3), Melanoma differentiation association protein 5 (MDA5) and interferon β (IFN-β) occurred during virulent IBV infection of tracheal organ cultures or kidney cells (Chhabra *et al.,* 2016). Further understanding of the importance of how various innate response factors interact during IBV infection, particularly regarding apoptosis, will aid future IBV research. To date, studies utilising TOCs have produced results that association innate immune responses and apoptotic cells (Chhabra *et al.,* 2016).

The aim of this study is to examine the virulence of common and important live IBV vaccines in TOCs and to cross-compared it against the corresponding virulent strains. As increasing numbers of live IBV vaccines are available in UK and globally, we were aiming to develop an in-vitro model, whereby the virulence of several IBV vaccine viruses could be assessed concurrently.

6.2 Materials and methods

6.2.1 Viruses

To compare strain pathogenesis and host innate immune responses, three common virulent (793B, Massachusetts and QX) serotypes of IBV and vaccines strains from the same serotypes (Mass1, Mass2, Mass3, 793B1, 793B2, 793B3, QX1 and QX2) were used in this

experiment. Vaccines IBV strains were passaged one time in the eggs and titrated in the TOCs prepared from 19 to 20-day old SPF EGE chicks (Chapter 2.1)(Cook *et al.,* 1976b).

6.2. 2 Tracheal organ culture

Tracheal organ cultures (TOC) were prepared as described in details in (Chapter 2.2). Titration of vaccine and virulent viruses was carried out in TOCs following the method defined in (Chapter 2.2) (Cook *et al.,* 1976b).

6.2.3 Experimental design

Three virulent strains of IBV, Mass ($10^{6.33}$ CD50/ml), 793B ($10^{6.38}$ CD50/ml) and QX ($10^{6.39}$ CD50/ml) which were previously grown in embryonated SPF chicken eggs and titrated in TOCs (Chapter 2.2).

Eight commercial vaccines, Mass1 ($10^{4.75}$ CD50/ml), Mass2 ($10^{6.5}$ CD50/ml), Mass3 ($10^{5.75}$ CD50/ml), 793B1 ($10^{7.25}$ CD50/ml), 793B2 ($10^{5.75}$ CD50/ml), 793B3 ($10^{4.5}$ CD50/ml), QX1 ($10^{5.75}$ CD50/ml) and QX2 ($10^{4.4}$ CD50/ml) were used in this study. All commercial vaccines were reconstituted in TOC media according to manufacturer's instructions. For each vaccine, 0.1 ml of reconstituted vaccine was separately inoculated into the allantoic cavity of 10 SPF eggs and incubated for 48h at 37°C (Dhinakar Raj and Jones 1996; Raj and Jones 1997a) , then the collected allantoic fluids were titrated in TOCs (Chapter 2).

Under sterile conditions, tracheas were removed and cut into 0.6-mm-thick rings using a tissue chopper[48] and individually transferred into sterile tubes containing 0.6 ml of TOC

[48] McIlwain (Mickle Laboratory Engineering)

medium (Chapter 2.2). Tubes were incubated at 37°C in a rotating incubator (Ball *et al.*, 2016b; Chhabra *et al.*, 2016).

Over five-days of period, cilia motility was checked for eight times (8, 24, 32, 48, 56, 72, 96, 120 hours post infection), and 15 samples per time point for each strain were collected at 24 and 72 hpi (Dhinakar Raj and Jones, 1996). For the entire experimental period, mock infected TOC tubes were included as control, for cilia motility readings and tissues for host gene expression or counting apoptotic cells. TOC medium collected at sampling time points was used for molecular analysis and were stored at -70°C. Tracheal rings were stored in 200 µl RNA*later*® (Qiagen, UK) at -70°C. Ciliary beating was scored as follows: 4= 0% beating; 3= 1-25% beating; 2= 26-50% beating; 1= 51-75% beating; 0= 76-100% beating (Cook *et al.*, 1999; Ball *et al.*, 2016b). The ciliostasis 50% end point is the time needed for the epithelial cells to show half of its cilia were beating (cilia viability).

Comment [GK7]: You must consult Chris on calculation time taken for 50% RCA!!!.

6.2.4 Virulent and vaccine viruses' infection of TOCs

The prepared TOCs were inoculated five days post infection, during this period signs and symptoms associated with the inflammatory response subside (Reemers *et al.*, 2009a). For all virulent and vaccine strains, a dose of 10^2 CD_{50}/ml was chosen based on preliminary data for which this titre caused 100% ciliostasis in TOCs by 72 hours post infection (hpi).

At each sampling point, five rings in RNAlater were used for each IBV strain and the mock infected group. Cilia activity in the TOCs was measured at 8, 24, 32, 48, 56, 72, 96 and 120 hour post infection (hpi) under an inverted microscope to determine the relative ciliary activity (RCA). Tracheal rings and supernatant were collected at 24 and 48 hpi for conventional RT-PCR and sequencing, quantification of infectious virus, TOC rings for quantification of host gene expression analysis. Using 4% paraformaldehyde (PFA) for fixation, TOC rings were evaluated for apoptotic cells using *in situ* Terminal

deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay (Chhabra *et al.*, 2016).

6.2.5 Ciliostasis quantification

Before infection, TOCs used in this study had a relative ciliary activity (RCA) of 100%. Ciliary vigour was measured at each time point as follows: 0 for lack of beating, 1 for slow in co-ordinated beating, 2 for moderate co-ordinated beating and 3 for fast harmonic beating (Gabridge *et al.*, 1974; Stadtlander *et al.*, 1991). The ciliostasis 50 % was analysed by multiplying the RCA with ciliary vigour. The time taken, in day post infection (dpi), for IBV strains to cause a 50% reduction in ciliary activity was calculated by polynomial regression (Raj and Jones 1996b)

6.2.6 Extraction of RNA

6.2.6.1 TOC media:

The total RNA from TOC media was extracted using the RNeasy Mini kit (Qiagen) (Chapter 2.13). Extracted RNA was quantified using a Nano-Drop 1000 spectrophotometer and stored at -70^{o}C until used for quantitative reverse transcription PCR (qRT-PCR) analysis (Chhabra 2016).

6.2.7 TOC rings:

In brief, the total RNA from five TOC rings was extracted using an RNeasy Plus Mini-QIA Kit (Qiagen, UK) following the manufacturer's instructions. The concentration of extracted RNA was quantified by Nano-Drop ND-1000 [49] (Chapter 2.14).

[49] Labtech international

6.2.8 Conventional IBV RT-PCR partial S1

IBV RT-PCR was conduct as previously described (Worthington *et al.,* 2008) that targeted a 393bp portion of the S1 gene (Chapter 2.15). Positive amplicons were sent for commercial Sanger sequencing using the forward SX3+ primer (Chapter 2.15.5).

6.2.9 Quantification of IBV RNA

At sampling points (0, 24, 72 and 120 hpi), qRT-PCR viral load was quantified using Rotor Gene Probe RT-PCR (Qiagen, UK). All the reagents and times are illustrated in details in (Chapter 2.18) as described previously (Roh *et al.*, 2014).

6.2.10 Host gene expression analysis

The transcription of innate immune genes, such as IFN-α and IFN-β TLR3 and MDA5 were nominated based on previous experimental results (Chhabra *et al.,* 2016). Quantitative reverse transcription PCR (qRT-PCR) of cDNA was conducted in triplicate from each sample using a Light Cycler® 480. The details on this procedure and primers are illustrated in Chapter 2.19.

6.2. 11 Detection of apoptotic cells in TOCs

Apoptotic cells were identified by the TUNEL assay using the peroxidase (POD) *in situ* cell death detection kit with Fluorescein according to the manufacturer's instructions (Roche, sigma-aldrich). In brief, tracheal rings were taken at 24 and 48 hpi and fixed in 4% PFA, for de-waxing, the TOC sections were incubated with 0.1 % Triton X-100 in 0.1% sodium citrate for 8 min at room temperature. Then PBS was used for washing 3 times, at that time sections were overlaid with 50 µl TUNEL reaction mixture and incubated in a humidified atmosphere for 60 min at 37°C in the dark. After repeating the washing step with PBS, 50 µl Converter-POD was added and the slides were incubated in a humidified atmosphere for 1 hour at 37°C. The washing step was repeated and the peroxidase reaction

was developed using the DAB Peroxidase Substrate kit according to the manufacturer's instructions. Haematoxylin counter stain was applied to the sections for 1 min and mounted with Aquatex. Five sections were analysed for each time point and calculation the average total apoptotic cells.

6.2.12 Statistical analysis

Analysis of 50% ciliostasis endpoints (50%EP) was carried out according to the established protocol (Raj and Jones 1996b). Data from the TUNEL assay and gene expression analysis were analysed by ANOVA using Graph Pad Prism, then the post-hoc LSD multiple comparison test. Differences between groups at that time point were considered significant at $P<0.05$.

Comment [GK8]: You mentioned about RCA before (above). This is what you suppose to follow throughout the chapter.

For cilia beating, how long they survive may not give sufficient information on virulence!

6.3 Results

6.3.1 TOCs reading

6.3.1.1 Complete ciliostasis reading

In the control group: there was no ciliostasis at the time of the end of the experiment at 120 hpi.

Virulent strains comparsion: VirMass showed the shortest time needed to reach complete ciliostasis at 72 hpi, earlier than both Vir793B and VirQX which achieved complete ciliostasis 120 hpi.

In the Mass group serotype comparison: VirMass showed complete ciliostasis earlier than other vaccines at 72 hpi. Mass1 showed complete ciliostasis later at 96 hpi, and both Mass2 and Mass3 shown complete ciliostasis later still at 120 hpi.

In the 793B group serotype comparison: All strains (Vir793B, 793B, 793B2 and 793B3) presented with complete ciliostasis at 120 hpi.

In the QX group serotype comparison. Both strains (VirQX and QX1) presented with complete ciliostasis at 120 hpi, while even at 120 hpi, QX2 still retained some viable cilia.

Vaccines strains comparisons: Mass1 showed an early time to reach complete ciliostasis at 96 hpi in comparison to other vaccines used in this study which showed complete ciliostasis at 120 hpi. Only QX2 showed some viable cilia at 120 hpi.

6.3.1.2 Ciliostasis end point 50% time point

The ciliostasis EP values of 50% between the three serotypes gave no statistical differences in their average values (Figure 6.1) (a lower value would mean more virulent, faster damage to the TOCs).

Data for the 50% EP within Mass group revealed statistical significant differences ($P<0.05$). In both VirMass (23.8 hpi) and Mass2 (24 hpi), there was significantly less ciliostasis ($P<0.05$) shown by 50% EP when compared to Mass1 (36 hpi), and Mass3 (34 hpi).

In the 793B group, the 793B3 strain displayed a late 50% EP at 39 hpi in comparison to the 793B1 (24.1 hpi) and 793B2 (23.9 hpi). In the QX group, there were significantly earlier differences ($P<0.05$) in 50% EP in both VirQX (24.5 hpi) and QX1 (28.3 hpi) when compared to QX2 which reached 50% EP at (46.5 hpi).

Comparisons among vaccines of different serotypes showed that Mass2, 793B1, 793B2 and QX1 had a significantly ($P<0.05$) lower 50%EP than Mass1, Mass3, 793B3 and 793B2 (Figure 6.1).

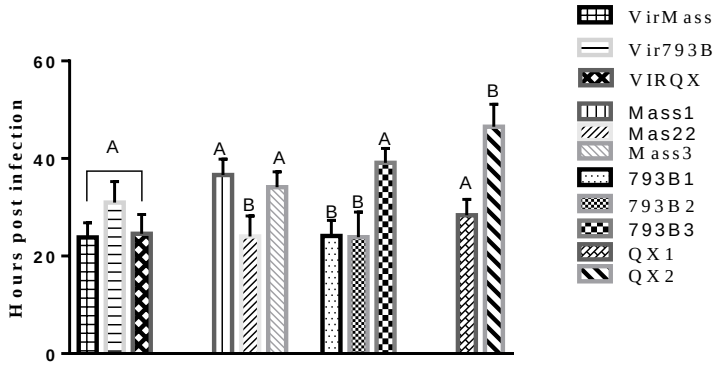

Figure 6.1. The 50% RCA end point values comparison between each serotype. For each strain, average 50% ciliostasis threshold values, representative of readings of 8 tubes each, were used to calculate the mean value of the strain. Significantly different ($p<0.05$) values are shown with different letters.

6.3.2 RT-PCR and SNP analysis

Media samples from 24 and 72 hpi were tested with conventional RT-PCR and sent for sequencing to be compared with the original inoculum sequences. No mutations, deletions or insertions occurred with the exception of one strain (Figure 6.2).

The virulent 793B inoculum had one sample at 72hpi with two single nucleotide polymorphisms (SNPs), located at 97 (Glycine to Alanine) at the position 856 bp (full S1) and 225 (Threonine to Alanine) positions at the position 1013 bp (full S1), that trigger amino acid changes (from Aspartic acid to Asparagine in the first case, from Phenylalanine to Leucine in the second one). However, there was no suspected change to the protein structure, as there was no hydrophobicity change to the amino acids (Aspartate and Asparagine polar, Phenylalanine and Leucine nonpolar).

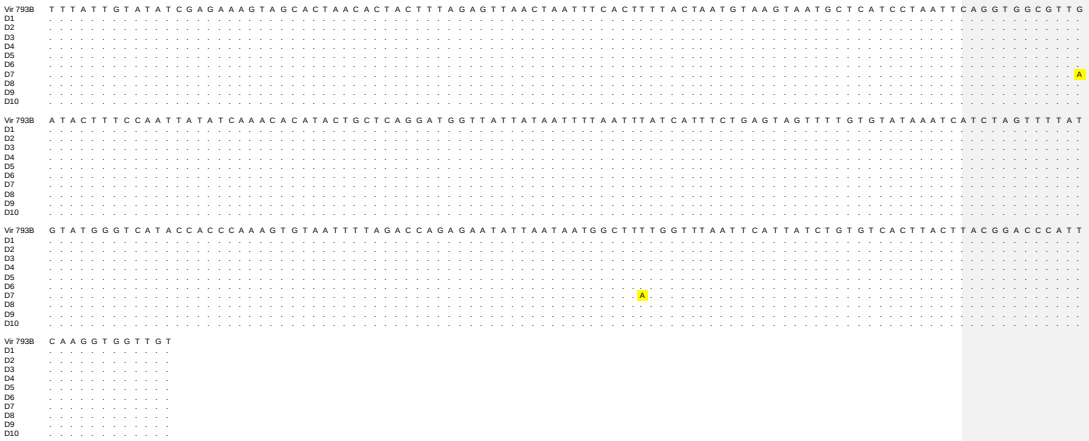

Figure 6.2. DNA sequence alignment of the partial S1 hypervariable region for vir793B. Lines D1 to 5 correspond with 24hpi samples, D6 to 10 correspond with 72 hpi samples. The starting position at 756 bp in the full S1, highlighted letters indicated changing in the nucleotides.

6.3.3 Viral RNA Quantification using qRT-PCR

6.3.3.1 Comparison over the incubation period

There were significant differences among strains at ($P<0.05$) for all three time points (Figure 6.3). Viral load of different strains of IBVs showed significantly difference values between 0 and 24 hpi, and 793B1, 793B2, 793B3 and QX1 had significantly ($P<0.05$) lower values at 72 hpi when compared to 24 hpi. There were significantly ($P<0.05$) lower differences at 72 hpi when compared to 0 hpi in all strains.

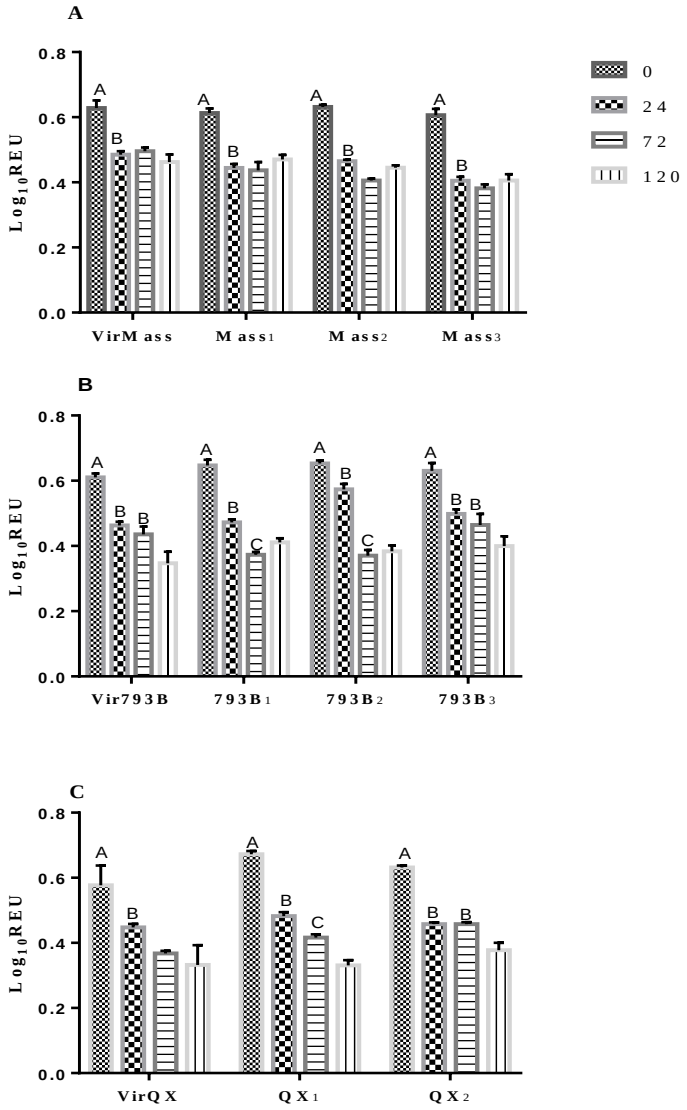

Figure 6.3. Quantification of viral load, values are shown in log REU (relative equivalent unit). Data from the three groups (A) Mass, (B) 793B and (C) QX group's qRT-PCR time point comparison. Significant differences between the groups were detected by one-way ANOVA and values ($P<0.05$) are shown with different letters.

6.3.3.2 Comparison between strains:

In the Mass serotype, at 24 hpi, there was a significantly ($P<0.05$) higher viral load in VirMass and Mass1 in comparison to the Mass2 and Mass3 serotypes. At 72 hpi, there was a significantly ($P<0.05$) higher viral load in VirMass in comparison to Mass1, Mass2 and Mass3.

In the 793B serotype, Vir793B, at 24 hpi, there was a significantly ($P<0.05$) lower viral load in comparison to 793B1, 793B2 and 793B3. At 72 hpi, there were significantly ($P<0.05$) higher viral load in Vir793B and 793B3 in comparison to 793B1 and 793B2.

At 72 hpi, in the QX serotype VirQX, there was a significantly ($P<0.05$) lower viral load in comparison to QX1 and QX2. At 72 hpi, there was a significantly ($P<0.05$) higher viral load in QX2 in comparison to QX1.

6.3.4 Apoptotic cells in TOC rings

Results for virulent or vaccine infected TOC rings are illustrated in Figure 6.4. Total apoptotic cells following infection with different IBV serotypes and differences between vaccines and virulent strains within the same serotype or mock infected TOCs were evaluated by TUNEL assay (Figure 6.5).

At 24 hpi, there was a significant difference ($P<0.05$) in VirM41 in comparisons to other vaccine strains (Mass1, Mass2 and Mass3). No significant differences found between the Mass1, Mass2 and Mass3 and mock infected control group or between these strains.

At 72 hpi, in the Mass serotype group, there was a significantly difference ($P<0.05$) in VirM41 in comparisons to the mock infected control group. There was a significant difference ($P<0.05$) in VirM41 in comparisons to other vaccine strains (Mass1, Mass2 and

Mass3). No significant differences between Mass strains Mass1, Mass2 and Mass3 and mock infected control group occurred. There were no significant differences between Mass strains Mass1, Mass2 and Mass3. At 24 hpi and 72 hpi, the highest average number of apoptotic cells was found in the Mass1 group.

At 24 hpi, in the 793B serotype group, there was a significant difference ($P<0.05$) in Vir793 in comparisons to the mock infected control group. There was a significant difference ($P<0.05$) between Vir793B and other vaccine strains (793B1, 793B2 and 793B3). No significant differences occurred between 793B1, 793B2 and 793B3 and the mock infected control group or between these vaccine strains.

At 72 hpi, in the 793B serotype group, there was a significant difference ($P<0.05$) between Vir793 and the mock infected control group, and the 793B vaccine strains (793B1, 793B2 and 793B3). There were no significant differences between 793B1, 793B2 and 793B3 and the mock infected control group or between these vaccines. At 24 hpi and 72 hpi, strain 793B1 had the highest average number of apoptotic cells among the vaccine strains.

In the QX serotype group, at 24 hpi, there was a significant difference ($P<0.05$) in VirQX in comparisons to the mock infected control group, and against the QX vaccine strains (QX1 and QX2). No significant differences occurred between QX1 and QX2 and the mock infected control group.

At 72 hpi, in the QX serotype group, there was a significantly higher difference ($P<0.05$) in VirQX in comparison to the mock infected control group, also against the QX vaccine strains (QX1 and QX2).

Cross-comparisons indicated that there were significantly higher differences (P<0.05) at 24 and 72 hpi between the three virulent serotypes (VirMass, Vir793B, VirQX) in comparison to respective vaccines within the same serotype (Figure 6.4).

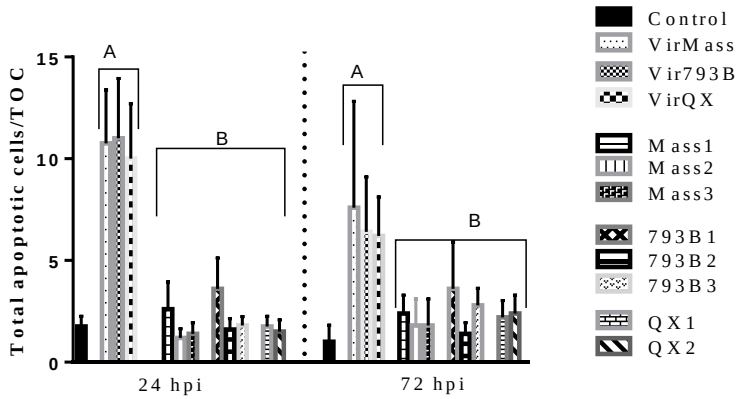

Figure 6.4. Total apoptotic cells in TOCs evaluated by TUNEL assay. TOCs were infected with IBV variant M41, 793B and QX, or with vaccine based from the virulent strains, or mock infected and evaluated at 24 and 72 hpi. Data represents mean with error bar showing standard error. Differences between the groups were detected by one-way ANOVA, followed by the post-hoc LSD multiple comparison test. Significant differences are indicated with different letters (P<0.05).

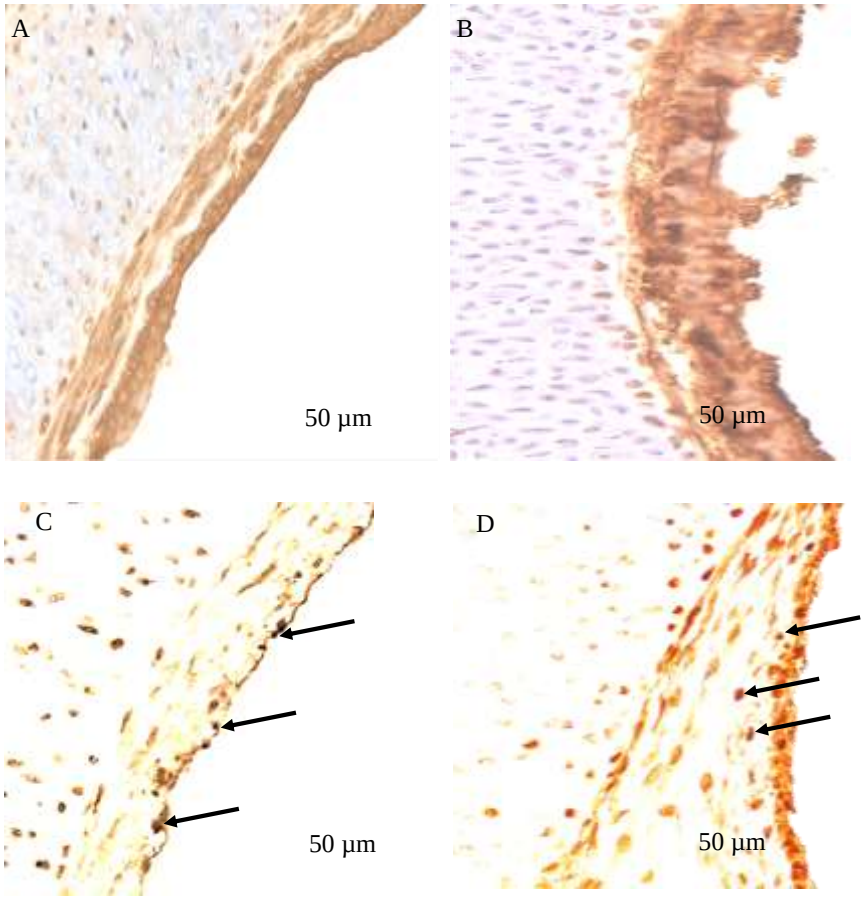

Figure 6.5. In situ Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining of TOCs; (A) Mock infected 24 hpi, (B) Mock infected 72 hpi, (C) VirQX infected 24 hpi or (D) VirMass infected at 24 hpi. Apoptosis TUNEL staining of TOCs infected with IBVs show multiple apoptotic cells with degeneration and loss of cilia. Significant differences between the groups were detected by one-way ANOVA. Arrows indicate apoptotic cells.

6.3.5 Host gene expression analysis

6.3.5.1 Relative IFN-α and IFN-β mRNA expression

6.3.5.1.1 Within each serotype mRNA IFN-α expression comparison

In the Mass serotype. At 24 hpi, there was significant (P<0.05) down regulation of the mRNA expression of IFN-α in the Mass1 strain in comparison to the control. There was significant (P<0.05) down regulation of the mRNA expression of IFN-α in the Mass1 strain in comparison to VirMass, Mass2 and Mass3. At 72 hpi, there were significant (P<0.05) down regulations of the mRNA expression of IFN-α in the VirMAss, Mass1, Mass2 and Mass3 strains in comparison to the control group (Figure 6.6A).

In the 793B serotype. At 24 hpi, there were significant (P<0.05) down regulations of the mRNA expression of IFN-α in the Vir793B, 793B1, 793B2 and 793B3 strains in comparison to the control group. There were significant differences (P<0.05) within 793B strains; Vir793B, 793B1 and 793B3 that showed significantly (P<0.05) lower differences in comparison to 793B2 (Figure 6.6B). At 72 hpi, there were significant (P<0.05) down regulations of the mRNA expression of IFN-α in the 793B1, 793B2 and 793B3 strains in comparison to the control group. There were significant differences (P<0.05) within 793B strains; 793B1, 793B2 and 793B3 that showed significantly (P<0.05) lower differences in comparison to Vir793B (Figure 6.6B).

In the QX serotype. At 24 hpi, there were significant (P<0.05) down regulations of the mRNA expression of IFN-α in the VirQX, QX1 and QX2 strains in comparison to the control group. There was significant (P<0.05) down regulation of the mRNA expression of IFN-α in the QX2 strain in comparison to the VirQX and QX1. At 72 hpi, there were significant (P<0.05) down regulations of the mRNA expression of IFN-α in the VirQX, QX1 and QX2 strains in comparison to the control group (Figure 6.6C).

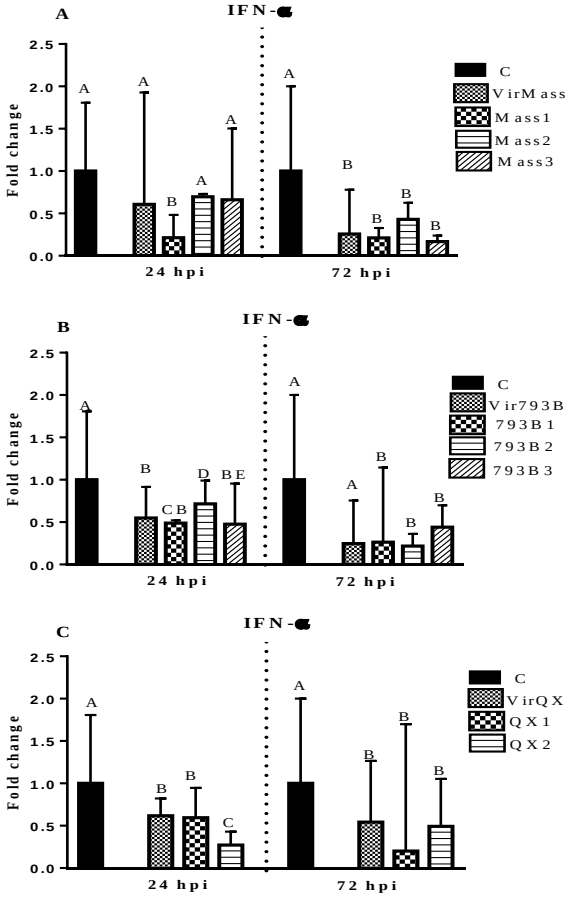

Figure 6.6. mRNA expression of IFN-β within each of the genotype. (A) Mass group, (B) 793B group and (C) QX group. Relative mRNA expression was determined by real-time PCR normalised to 18S mRNA. Graph values are the mean of three biological replicates with error bars showing standard error and are expressed as fold change relative to the mocked-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the post-hoc LSD multiple comparison test. Letters indicate significant differences ($P<0.05$).

6.3.5.1.2 Within each serotype mRNA IFN-β expression comparison

In the Mass serotype. At 24 hpi, there were significant (P<0.05) up regulations of the mRNA expression of IFN-β in the VirMass, Mass1, Mass2 and Mass3 strains in comparison to the control group. There was a significantly (P<0.05) higher difference in the VirMass mRNA IFN-β expression in comparison to Mass1. At 72 hpi, there were significant (P<0.05) down regulations in the mRNA expression of IFN- β in the VirMass, Mass2 and Mass3 in comparison to Mass1 (Figure 6.7A).

In the 793B serotype. At 24 hpi, there were significant (P<0.05) up regulations of the mRNA expression of IFN-β in the Vir793B, 793B1, 793B2 and 793B3 strains in comparison to the control group. There were significant higher differences (P<0.05) in 793B1 in comparison to the Vir793B and 793B3 groups. At 72 hpi, there was a significant (P<0.05) down regulation in the mRNA expression of IFN-β in the 793B1 group in comparison to the control group (Figure 6.7B).

In the QX serotype. At 24 hpi, there were significant (P<0.05) up regulations of the mRNA expression of IFN-β in the VirQX and QX1 strains in comparison to the control group. There was significant (P<0.05) down regulation of the mRNA expression of IFN-β in the QX2 strain in comparison to the VirQX and QX1. At 72 hpi, there was significant (P<0.05) down regulation of the mRNA expression of IFN-β in comparison to the control group. There was significant (P<0.05) down regulation of the VirQX in comparison to QX2 (Figure 6.7C).

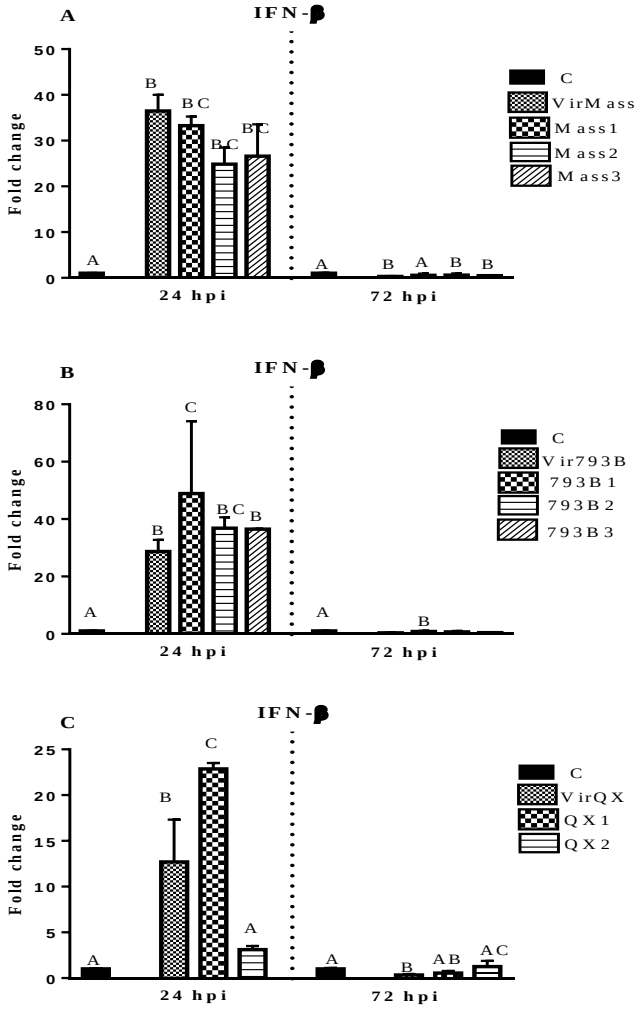

Figure 6.7. mRNA expression of IFN-β within each genotype. (A) Mass group, (B) 793B group and (C) QX group. Relative mRNA expression was determined by real-time PCR normalised to 18S mRNA. Graph values are the mean of three biological replicates with error bars as standard error which are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the post-hoc LSD multiple comparison test. Significant differences ($P<0.05$) are indicated with letters.

6.3.5.2 Expression of mRNA of TLR3 and MDA5

6.3.5.2.1 Within each serotype mRNA of TLR3 expression comparison

In the Mass serotype. At 24 hpi, there were significant ($P<0.05$) up regulations of the mRNA expression of TLR3 in the VirMass, Mass1, Mass2 and Mass3 strains in comparison to the control group. There were significantly ($P<0.05$) higher differences in the Mass2 and Mass3 TLR3 expression in comparison to VirMass. At 72 hpi, there were significant ($P<0.05$) up regulations in the mRNA expression of TLR3 in the VirMass, Mass1, Mass2 and Mass3 in comparison to the control group (Figure 6.8A).

In the 793B serotype. At 24 hpi, there were significant ($P<0.05$) up regulations of the mRNA expression of TLR3 in the Vir793B, 793B1 and 793B2 strains in comparison to the control group. At 72 hpi, there were significant ($P<0.05$) up regulations in the mRNA expression of TLR3 in the Vir793B, 793B1, 793B2 and 793B3 strains in comparison to the control group (Figure 6.8B).

In the QX serotype. At 24 hpi, there were significant ($P<0.05$) up regulations in the mRNA expression of TLR3 in the VirQX, QX1and QX2 strains in comparison to the control group. At 72 hpi, there was a significant ($P<0.05$) up regulation in the mRNA expression of TLR3 in comparison to the control group. There were significantly ($P<0.05$) higher differences in the QX2 in comparison to VirQX and QX1. There was a significantly ($P<0.05$) higher differences in the QX1 in comparison to the VirQX (Figure 6.8C).

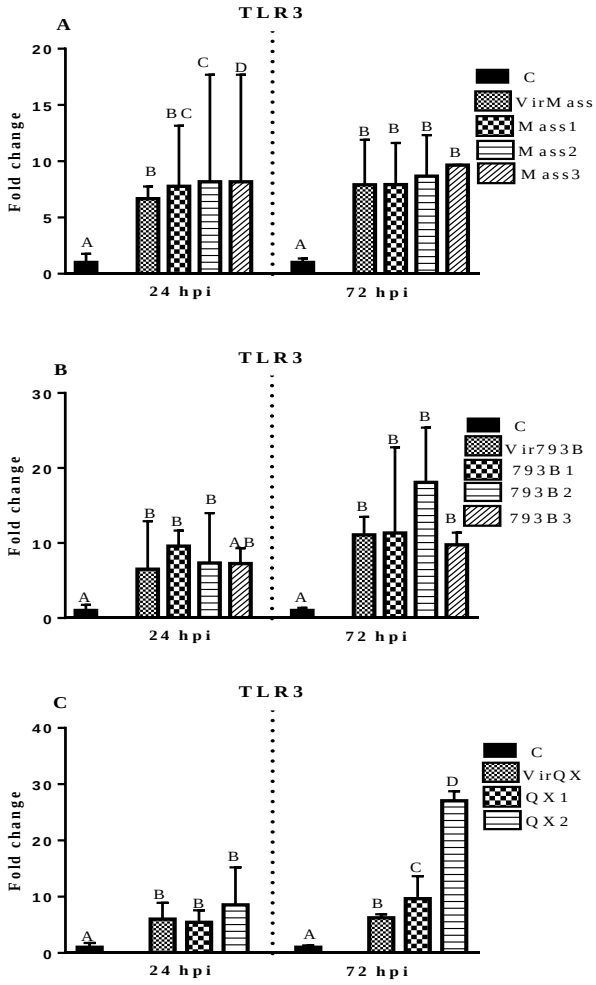

Figure 6.8. mRNA expression of TLR3 within each genotype. (A) Mass group, (B) 793B group and (C) QX group. Relative mRNA expression was determined by real-time PCR normalised to 18S mRNA. Graph values are the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the post-hoc LSD multiple comparison test. Significant differences ($P<0.05$) are indicated with letters.

6.3.5.2.2 Within each serotype mRNA of MDA5 expression comparison

In the Mass serotype. At 24 hpi, there were significant (P<0.05) up regulations of the mRNA expression of MDA5 in the VirMass, Mass1, Mass2 and Mass3 strains in comparison to the control group. There were significantly (P<0.05) higher differences in the VirMass and Mass1 in comparison to Mass2 and Mass3. At 72 hpi, there was significant (P<0.05) up regulation in the mRNA expression of MDA5 in Mass1 in comparison to the control group (Figure 6.9A).

In the 793B serotype. At 24 hpi, there were significant (P<0.05) up regulations of the mRNA expression of MDA5 in the Vir793B and 793B1 in comparison to the control group. There were significantly (P<0.05) higher differences in the mRNA expression of MDA5 in the Vir793B and 793B1 in comparison to 793B2 and 793B3 strain groups. At 72 hpi, there was a significantly (P<0.05) higher differences in the mRNA expression of MDA5 in 793B2 in comparison to 793B3 (Figure 6.9B).

In the QX serotype. At 24 hpi, there were significant (P<0.05) up regulations of the mRNA expression of MDA5 in the VirQX, QX1and QX2 strains in comparison to the control group. There was a significantly (P<0.05) higher difference in the QX1 in comparison to the QX2 (Figure 6.9C).

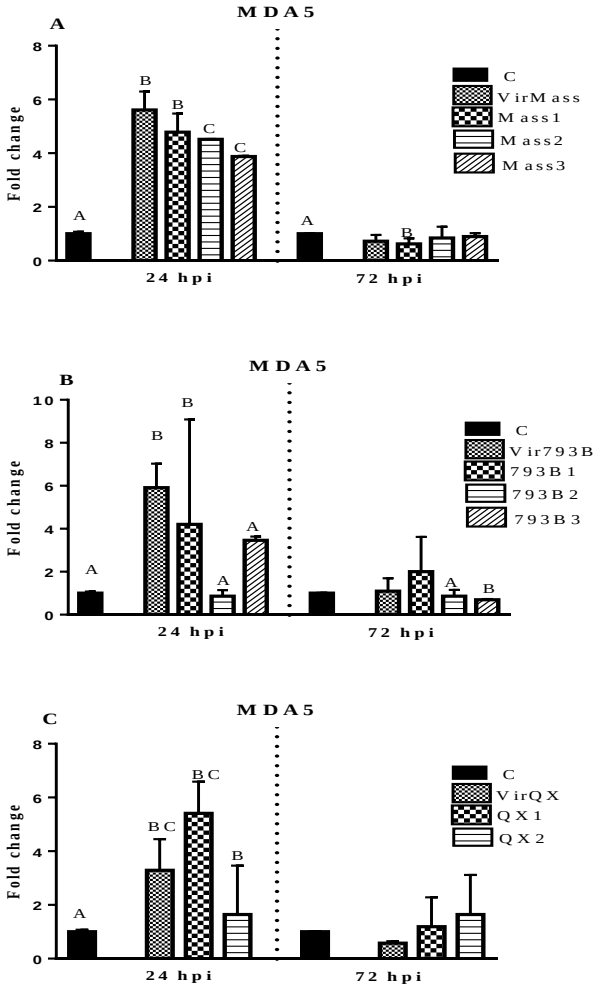

Figure 6.9. mRNA expression of MDA5 within each genotype. (A) Mass group, (B) 793B group and (C) QX group. Relative mRNA expression was determined by real-time PCR normalised to 18S mRNA. Graph values are the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the post-hoc LSD multiple comparison test. Significant differences ($P<0.05$) are indicated with letters.

6.4 Discussion

In this experiment, the *in vitro* system (TOCs) was used to an attempt to find a possible *in vitro* model for comparison of live IBV vaccines, where a number of IBV vaccines can be simultaneously cross-compared. Major live IBV vaccines belonging to three main serotypes, Mass, 793B and QX from different producers were included in this study. In addition, virulent IBVs belonging to the same serotype were also included for comparison. A number of parameters were examined including ciliostasis, time taken to reach 50% RCA, quantification of IBV replication, apoptosis and host innate immune responses, including mRNA expression of interferons (IFN-α, IFN-β) and sensing molecules (TLR3 and MDA5). In this study, *in vitro* analyses on IBV-infected TOCs has illustrated a possible experimental model that could distinguish the virulence between live IBV vaccine viruses.

After the relative ciliary activity (RCA) analysis, data showed that VirMass has the earliest time to reach complete ciliostasis (72 hpi), and the time to reach 50 % RCA was 23.8 hpi. This finding broadly supports the work of other studies in this area linking the pathogenies of M41 by tropism of this virus with lesions or damage to the tracheal epithelia (Raj and Jones 1996b; Chhabra 2016). In all strains tested in this study, the most obvious finding to emerge from the analysis of RCA and time taken for all the TOC rings to have complete ciliostasis for QX2 was 46.5 hpi and >120 hpi respectively. Also, QX2 has the highest expression of TLR3 and the lowest expression of IFN-α and IFN-β. Such finding may be emphasise that QX variants have a lower replication rate compared to other serotypes (Benyeda *et al.*, 2009). As a result, it would take a relatively long time to start an effective infection, which is in agreement with previous findings which report that QX tends to infect the reproductive tract and kidney instead of the respiratory tract (Benyeda *et al.*, 2010; Chhabra *et al.*, 2016).

Comment [GK9]: This is very confusing….check with Chris or myself/

SEE FIGURE 6.1

In this study, the virulent strains (VirMass, Vir793B and QX) and Mass1, 793B1 and QX1 reached nearly the 50% time point at the same average time. During this experiment, at 24 hpi, a higher expression of MDA5 and IFN-β were found. According to these data, we can infer that the MDA5 and IFN-β are linked to the pathogenicity and tissue damage and could be used as a virulence indicator in TOCs.

The results of viral loads in all strains indicated that, there were lower quantities at 72 hpi, compared to the point of inoculation. This may be due to intracellular movement of viral particles from the media (where they were added for infection) into the tracheal cells. Moreover, epithelial cell death could occur, as apoptosis is initiated as a result from virus replication (Liu *et al.,* 2001; Fung *et al.,* 2014), and apoptosis progression could impair virus proliferation, effecting viral load (Chhabra *et al.,* 2016). A previous study has explored the relationship between viral titre and time in TOCs, and findings proved that viral titres reduced over the duration of the study; while the amount of viral genome increased (Ball *et al.,* 2016b).

IBV infection modulates apoptosis by restricting activation of the extracellular signal-regulated kinase pathway (Chhabra *et al.,* 2016). In addition, a strong association has been noted between apoptosis in TOCs and respiratory lesions in chickens (Li *et al.,* 2007). At 24 and 72 hpi, there were significant difference in the total number of apoptotic cells in TOCs infected with the virulent serotypes but no differences between vaccines. This is also in accordance with earlier observation, which showed that apoptosis is associated with viral virulence and pathogenicity in TOCs (Chhabra *et al.,* 2016). Notably, there were low numbers of apoptotic cells for all vaccine strains, which could be in high passage level or that vaccine strains need longer time or higher dosages to produce a greater number of apoptotic cells.

The expression of IFN-α showed a significant down-regulation in approximately all strains, this result is contradicted to previous study when TOCs infected with virulent M41, 885 and QX, (Chhabra *et al.,* 2016). Probably this is due to the fact that during infection of their host cells, IBVs deter the production of host proteins, in a process of shut off, which hampers host proteins, limits production of antiviral proteins and increases the capacity of viral proteins and type I interferon (Kameka *et al.,* 2014; Kint *et al.,* 2016). Such a shut off has an effect on the level of expression of the mRNA IFN-α during the early stages of infection. Coronaviruses are well known to down regulate host gene expression and increase host mRNA degradation in order to suppress host innate immune responses (Kamitani *et al.,* 2006; Tohya *et al.,* 2009). Results indicate that the virulent and vaccine strains used in this study are reducing production of IFN-α.

At 24 hpi, there was a significant up-regulation in the mRNA expression of IFN-β from both variant and vaccine strains. The profile of IFN-β expression is very similar in all our experiments to Chhabra *et al.,* (2016), where a higher level IFN-β expression was found in the early stages of infection after 9 and 24 hpi. Interferons can activate the up-regulation of TLR3, which, in some cell types, induces them to become responsive to dsRNA (Karpala *et al.,* 2008).

Within the sampling times, the expression of TLR3 was strongly up regulated in all virulent and vaccine serotypes. Increased activation of this gene in this study corroborates with earlier findings when M41, QX and IS/885/00 serotypes were tested (Chhabra *et al.,* 2016). It has been reported that TLR3 engagement is important to the outcome of viral infection because of its role in the induction of IFNs and the diverse antiviral effects that these molecules induce (Karpala *et al.,* 2008).

In the current study, our data showed that MDA5 mRNA expression, was significantly up regulated in VirMass, Mass1, Mass2, vir793B, 793B1, 793B3 and QX1 strains at 24 hpi. However, at 72 hpi, it was also significantly up regulated in 793B1 in comparison to the control and other strains. Similar increases have been reported before (Chhabra *et al.*, 2016). MDA5 has been reported to inhibit the growth of vesicular stomatitis virus and encephalomyocarditis virus (Yoneyama *et al.*, 2005), and in influenza A virus-infected epithelial cells, it has also been demonstrated that expression of MDA5 lead to the stimulation of the IFN-β (Siren *et al.*, 2006).

Considering our data, we observe there was a robust relationship between expression of TLR3, IFN-β and MDA5 for both variant and vaccine infected TOCs. Further studies are needed to strengthen this relationship, in conjunction with *in vivo* work to establish other pathways for determining the innate responses towards IBV infection of differing virulence. In this study there is an association between MDA5 and IFN-β with the 50% RCA time point, and apoptosis, where all were associated with increase in virulence. The opposite results were found in the QX2 strain, which has the longest time to reach the 50% RCA endpoint, showing no change in IFN-β expression and less expression of MDA5 within QX serotype. It is therefore likely the association between MDA5 and IFN-β with the cilisostasis of TOCs.

In conclusion, it appears that IBV infection of TOCs was a representative *in vitro* method for exploration of the pathogenesis of IBV infection, and it is a valuable in differentiating virulence between the IBV viruses. The use of TOCs for an IBV pathogenesis study has been shown to be practical and beneficial to reduce costs and time. In addition, the numbers of birds used for *in vivo* studies can be markedly reduced. The TOC is applicable and analogous to *in vivo* work, helping to reduce the number of required animal model experiments and work towards the 3Rs (Replacement, Reduction and Refinement).

Chapter 7: Assessment of virulence and innate immune responses following administration of infectious bronchitis vaccine viruses in specific pathogen free chicks

Abstract

Following inoculation of different infectious bronchitis virus (IBV) vaccine strains in specific pathogen free (SPF) chicks, the pathobiology was evaluated in conjunction with the host immune responses (innate, local, humoral and cellular). Four attenuated live IBV vaccines (Mass1, Mass2, 793B1 and 793B2) were administered by the oculo-nasal route and chicks were monitored daily for clinical signs. At 1, 3, 5, 7, 9 and 14 days post infection (dpi), oropharyngeal (OP) and cloacal (CL) swabs were collected for virus detection, and blood was collected for antibody quantification. Necropsy was carried out on 1, 3. 5, 7, 9 and 14 dpi, with gross lesions observed and tracheal tissues collected for qRT-PCR, CD4+:CD8+ ratio, immunohistochemistry, histopathology and innate immune gene expressions. Variations in the virulence were found. Clinical signs were found in all vaccinated groups. Viral load was lower in the 793B1 group at 5-7 dpi, compared to other groups. Higher expression of IFN-β was seen at 3 dpi in 793B groups, whereas793B2 showed a lower expression of TLR3 at 5-7 dpi compared to other groups. Down-regulation of IL-6 was seen at 7-9 dpi in all inoculated groups (not Mass1). Lachrymal IgA increased at 14 dpi in all groups. There was higher up-regulation of IFN-γ at 7-9 dpi in Mass1 and Mass2 compared to the control and 793B1 and 793B2. There was higher up regulation in IgG expression in the Mass1 and 793B at 7-9 dpi. The 793B2 inoculated groups demonstrated higher cell mediated immune responses, represented by higher CD8β gene expression in the period of 5-9 dpi, higher counting of CD4+ and CD8+ at 14 dpi, and higher CD4:CD8 ratio at 7 and 14 dpi.

7.1 Introduction

Since the first vaccine was developed against Massachusetts serotype, a number of other vaccines from other serotypes were introduced for higher and better protection against classical and variant IBVs. The most widely used live IBV vaccines, such as H52/H120/Ma5 are derived from the Massachusetts serotype (Bijlenga *et al.*, 2004; Jackwood and de Wit 2013; Zhou *et al.*, 2016). Other common live vaccines belonging to Dutch (eg. D274), 793B (e.g. 4/91, CR88 and 1/96) and QX strains have been reported in poultry-producing countries (Jones 2010; Ball *et al.*, 2016a). Vaccination is an efficient way to prevent and control disease and production losses caused by IBV infection. Following vaccination large numbers of T and B cells and plasma cells are found in the trachea (Matthijs *et al.*, 2009). Responses in the lung and upper and lower trachea are comparable and do not seem to be location dependent (Reemers *et al.*, 2009b). Many researchers outlined distinct categories of innate immune response often observed in either IBV vaccination or infection, such as IFNs, TLRs, MDA5, and pro-Inflammatory cytokines (Okino *et al.*, 2013; Śmiałek *et al.*, 2016; Okino *et al.*, 2017; Chhabra *et al.*, 2018).

There is a growing body of literature that recognises the importance of local and cellular immune responses in the trachea and lachrymal fluid after IBV vaccine inoculation, either with or without challenge. It has been shown that such immune responses are dose dependant (Okino *et al.*, 2013). No previous research has been conducted to monitor a number of vaccine strains from the same serotype, particularly measuring the local, cellular and humoral immunity at the molecular level (Okino *et al.*, 2013; Zhang *et al.*, 2017). This study was carried out to examine the host responses of tracheal tissue, tracheal washes and

lachrymal fluid to inoculated vaccines (belong to Mass or 793B serotypes) based on monovalent vaccine application viruses in SPF chicks.

7.2 Materials and methods

.2.1 Vaccine viruses

In this experiment, four different vaccine strains belonging to two serotypes were used; Mass1, Mass2, 793B1, and 793B2.All four vaccines were passaged once in embryonated SPF eggs (Chapter 2) and titrated in TOCs (Cook *et al.,* 1976a) (Chapter 2.6). Titres were determined according to the Reed-Muench method (Reed LJ 1938) and expressed as cilliostasis dose 50 (CD_{50}) (Chapter 6).

7.2.2 Chicks

Day-old SPF chicks were hatched at Leahurst Campus, Liverpool University. All birds were housed in an animal house, reared on deep litter, and feed and drinking water were given *ad libitum* (Chapter 2.1).

7.2.3 Experimental design

Two hundred day-old SPF chicks were split into five groups (n=40 chicks/group) and vaccinated via the oculo-nasal route with $10^{4.5}$ CD_{50} as follows: Group 1 = Mass1, Group 2 = Mass2, Group 3 = 793B1, Group 4 = 793B2 and Group 5 = mock infected control group. All groups were maintained in separate, isolated rooms. Lachrymal fluid, blood and tracheal washes were collected from five birds on 0, 7 and 14 days post inoculation (dpi). At 1, 3, 5, 7 and 9 dpi the tracheas were collected for qRT-PCR and host gene analysis. The lachrymal fluid and serum samples were stored at -20 $^{\circ}$C and blood samples were processed immediately for peripheral blood mononuclear cells isolation. For immunohistochemistry, to count CD4+ and CD8+ cells, the middle section of the trachea

after post-mortem was placed in OCT and snap-frozen in liquid nitrogen. Another tracheal portion was collected in RNA*Later* and stored at -20 °C until further processing for detection of viral load and host innate genes.

From each group, lachrymal fluid was collected from five birds at each sampling interval. Approximately 90-100 µl of lachrymal per bird was collected in 0.5ml Eppendorf tubes using a micropipette and stored at -20 °C for later use.

7.2.4 RNA extraction

7.2.4.1 Tracheal tissue:

Samples were collected in RNA later[50] and stored at -20 °C. RNA extraction was carried out on 30 mg of trachea using the RNeasy Mini Kit[51] according to manufactures instructions for total RNA extraction (Chapter 2.14).

7.2.4.2 Tracheal washes and lachrymal fluid:

Viral RNA was extracted from 150µl of trachea washes after centrifugation at 3000 g for 10 min, using the QIAamp viral RNA Mini Kit (Qiagen, UK), according to the manufacturer's instructions. Lachrymal fluid was pooled and RNA extracted from 80-100 µl (Chapter 2.13).

7.2.5 IBV RT-PCR partial S1

IBV RT-PCR was conducted as previously described (Worthington *et al.*, 2008) (Chapter 2.15).

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[50] Qiagen

[51] Qiagen

7.2.6 Real-time RT-PCR

Quantification of extracted viral RNA was done by quantitative real-time RT-PCR (qRT-PCR) using IBV 3'untranslated region (UTR) gene-specific primers and probes as described previously (Jones et al., 2011) (Chapter 2.18).

7.2.7 Measuring IgA in the lachrymal fluid and mRNA expression of IgA from tracheal washes

Lachrymal fluid samples were centrifuged at 3000 g for 10 m and the supernatant were stored at -20°C until used for ELISA. Following the manufacturer's instructions, assessment of immunoglobulin A (IgA) in the lachrymal fluid was carried out using IgA chicken ELISA kit (Abcam, UK).

Samples were incubated for 10-15 m at room temperature, vortexed and diluted to 1:25. Each sample (100 µl) was added to a 96-well immunoplate, pre-coated with anti-IgA antibodies, and incubated for 20 minutes at room temperature (22-26°C). Then, unbound samples were removed in a wash step using PBS. Anti-chicken IgA antibodies conjugated with horseradish peroxidase (HRP) were then added to the plate and incubated for 20 min at room temperature, followed by another wash step. The enzyme complex was quantified by adding 3, 3', 5, 5' - tetramethylbenzidine (TMB) substrate (100 µl). The reaction was stopped by adding stop solution (100 µl) for 10 min and absorbance was measured at 450 nm using a multi plate reader. The quantity of IgA in the test sample was interpolated from a standard curve constructed from the standards, and corrected for sample dilution according to the manufacturer's guidelines.

Tracheal washes were collected at 7 and 14 dpi for measuring the IgA mRNA expression antibody titres. In brief, using two sterile artery forceps, clamped trachea was collected from glottis to syrinx and washed with PBS using 0.5-1 ml by syringe with 19 gauge

needle. The collected samples were centrifuged at 3000 x g for 5 min and supernatant was collected for RNA extraction QIAamp viral RNA Mini Kit (Qiagen, UK) (Ganapathy *et al.,* 2005).

7.2.8 Enzyme Linked Immunosorbent Assay (ELISA)

Sera samples were processed using a commercial IBV ELISA kit (IDEXX, USA) according to manufacturer instructions (Chapter 2.20). Sample/positive ratio (S/P) ratios were calculated and expressed as the mean serum anti-IBV antibody.

7.2.9 Host gene expression analysis

Samples of trachea was analysed for expression of mRNA of IFN-α, IFN-β TLR3, MDA5, IL-1β and IL-6, and results were compared against the control birds (mock infected), expressed as fold change relative to the 18S reference gene. Quantitative reverse transcription PCR (qRT-PCR) of cDNA was carried out using Light Cycler® 480 in triplicate (Table 7.1; Chapter 2.19). For IFNγ, CD8β, Myeloid differentiation primary response gene 88(MYD88), IgA and IgG, the calculation of fold change were carried out as previously described (Livak and Schmittgen 2001; Okino *et al.,* 2017), using 18S as a reference gene.

Table 7.1. Sequence of primers used in RT-qPCR for relative quantification of gene expression

Oligonucleotide	Sequence	Length	Reference
IFNγ(F)	5' AGCCGCACATCAAACACATA 3'	20	(Okino *et al.*, 2017)
IFNγ(R)	5' AAGTCGTTCATCGGGAGCTT 3'	20	
MYD88(F)	5' AGAGTTGGAGCAAACGGAGTTC 3'	22	(Okino *et al.*, 2017)
MYD88(R)	5' CATCCTCCGACACCTTCTTTCT 3'	22	
CD8β(F)	5' CTGCATGGCTCCGACAATGG 3'	20	(Okino *et al.*, 2017)
CD8β(R)	5' ATCGACCACGTCAAGCTGGG 3'	20	
ChIgA(F)	5'TGCAGGGCAATGAGTTCGTCTGTA 3'	24	(Ndegwa *et al.*, 2012)
ChIgA(R)	5'AGGAGGTCACTTTGGAGGTGAAT 3'	23	
ChIgG(F)	5 GACGAAGCTT TTCCTCTTCT'3'	20	(Zheng *et al.*, 2001)
ChIgG(R)	5' CCCGATTGTA CCCTCTATCG 3'	20	

7.2.10 Cellular immune response

7.2.10.1 T lymphocyte (CD4+:CD8a+) ratio from blood

Immediately after pentobarbitone intravenous injection, blood was collected from chicks in 5 ml tubes contain heparin[52] 10 USP/ml (United States Pharmacopeia) of blood. Once in the laboratory, it was diluted (1:1) with RPMI 1640 medium[53] (Roswell Park Memorial Institute). Subsequently, 1 ml of prepared blood was over layered on to 0.5 ml of Histopaque - 1.077 gradient[54] and centrifuged in a 1.5 ml Eppendorf tube for 90 sec at 10,000 rpm at room temperature. Mononuclear cells formed a buffy coat which was gently collected and washed twice with RPMI 1640 medium. Cells were re-suspended in 0.5%

[52] Sigma

[53] R8758, Sigma Aldrich

[54] 10771, Sigma Aldrich

Bovine serum albumin (BSA)[55] in PBS (blocking solution) and incubated at room temperature for 15 min and samples were allocated in 100µl. Samples were incubated with antibodies against surface domains at 0.2 µl per 100 µl of sample for 30 min in the dark. Targets included chicken CD4 (mouse anti-chicken CD4-FITC (Fluorescein Isothiocyanate) clone CT-4, 8210-02, Southern Biotech) and CD8 (mouse anti-chicken CD8a-FITC clone CT-8, 8220-02, Southern Biotech) receptors of T-lymphocytes (Appendix). After a final washing in PBS, the samples were fixed in 200 µl of 2% paraformaldehyde in PBS. The CD4+ and CD8+ lymphocytes were measured using a flow cytometer[56]. Negative unstained cells were used to adjust the threshold.

7.2.10.2 Detection of CD4+ and CD8+ cells by immunohistochemistry

Tracheal samples were cut into 5 µm sections on poly-l-lysine-coated glass slides[57] using a cryostat[58]. Sections were fixed in cold acetone for ten minutes and air dried for a further ten minutes. To each slide, 0.03% hydrogen peroxide in PBS was added for 20 minutes to inhibit endogenous peroxidase. Following inhibition, the VECTASTAIN Elite ABC kit [59] was used to block endogenous biotin or biotin-binding proteins. Then, mouse monoclonal antibodies against chicken CD4+ and CD8a at 1:1000 (Southern Biotechnology Associates

[55] A9418, Sigma Aldrich

[56] BD Accuri C6, San Jose, CA

[57] 631-0107, VWR International, Leuvan

[58]Leica CM 3050 S; Leica Microsystems, Heidelberger, Germany

[59] PK-6100, Vector Laboratories, Burlingame, USA

[60]) was added and incubated overnight at 4ºC in the dark to detect CD4+, CD8a+ cells. Slides were washed and avidin-biotinylated horseradish peroxidase reagent[61] was added to the slides for 30 minutes. Finally, peroxidase substrate kit DAB[62] was added to each slide, followed by a counterstain with haematoxylin[63] and mounted with DPX (dibutyl phthalate, polystyrene granules and xylene) neutral mounting medium. Five sections were randomly selected for counting positive stained cells under the light microscope (400 × magnifications). The final average was obtained for each slide after calculating the total positive cells/400× microscopic fields (Appendix).

7.2.11 Statistical analysis

Data were analysed using GraphPad Prism version 7 with one-way ANOVA, followed by the LSD for parametric data and Kruskal–Wallis followed by Dunn's tests for non-parametric data. Differences were considered significant at $p < 0.05$ (Chapter 2.22).

7.3 Results

7.3.1 Clinical signs and gross lesions

In the control group, there were no clinical signs observed during the period of the experiment. In all inoculated groups, there were mild respiratory signs, such as snicking and head shaking, starting from 3 dpi (more prominent in the 793B2 group) and lasted to the end of the experiment (Figure 7.1). There were no gross lesions in the control group,

[60] Birmingham, AL

[61] VECTASTAIN ABC kit

[62] SK-4100,Vector Laboratories, Burlingame, USA

[63] 1.09234, Merck, Germany

with only mild tracheal congestion seen for the inoculated groups (more prominent for the 793B2 group). At 9 dpi, for one bird in the 73B2 group, there was transparent serous to caseous exudate in the trachea and the infraorbital sinuses.

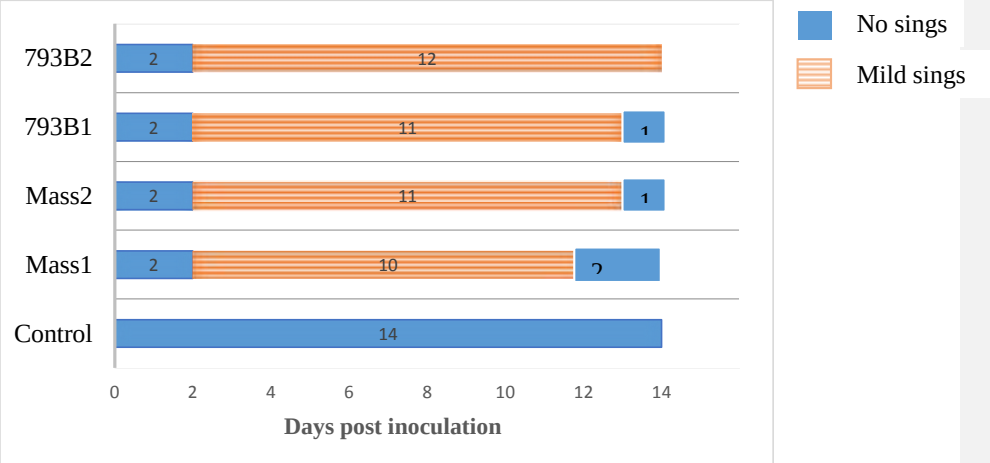

Figure 7.1. Onset and duration of clinical signs in the inoculated and control groups.

7.3.2 Histopathological changes

No histological changes were found in the control group. There were minor histopathological tracheal lesions in the inoculated groups, mainly consisting of a small amount of deciliation and lymphocyte infiltration. However, these changes were substantially greater in the Mass2 and 793B2 inoculated groups, especially at 5–14 dpi (Figure 7.2).

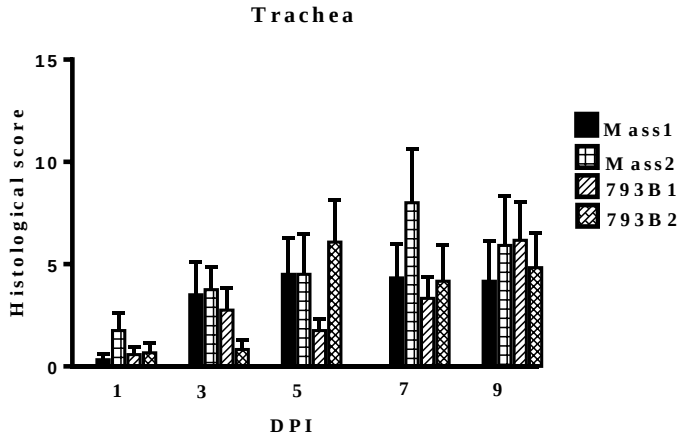

Figure 7.2. Means of histopathological scores of lesions in the trachea in the vaccinated groups. Significant differences between the groups were examined by the Kruskal–Wallis test, followed by Dunn's mean test ($p < 0.05$). There were no significant differences between the vaccine virus inoculated groups.

7.3.3. IBV virus detection from OP and CL swabs using RT-PCR

The virus was detected by RT-PCR in the OP and CL swabs from all vaccinated groups at all sampling days, except Mass1 and Mass2 at 1 dpi from CL swabs.

7.3.4. IBV virus detection from OP swabs using qRT-PCR

No virus detected in the control group. Viral load was at detectable levels from pooled OP swabs at all sampling days (1–9 dpi) using qRT-PCR in all inoculated groups. The Mass2 and 793B2 groups had the highest averages at 1 dpi, with quantifications decreasing on subsequent sampling points. Interestingly, viral load increased in all groups on 7 dpi, only to decrease again on 9 dpi (Figure 7.3).

Table 7.1. Virus detection by partial S1 RT-PCR from OP and CL swabs of inoculated groups on all sampling days and from both swabs

	Mass1		**Mass2**		**793B1**		**793B2**	
dpi	OP	CL	OP	CL	OP	CL	OP	CL
1	+	-	+	-	+	+	+	+
3	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+

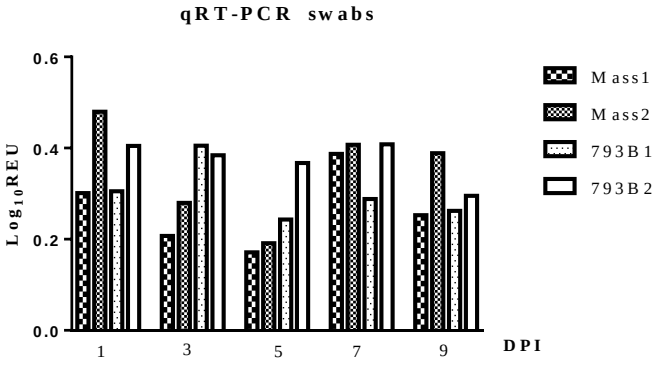

Figure 7.3. Quantification of infectious bronchitis virus (IBV) from OP swabs, expressed as log10 REU of RNA following infection in day-old chicks.

7.3.5 IBV virus detection from tracheal tissue using qRT-PCR

Viral RNA was detected in tracheal tissue for all sampling days; however, each group showed a different pattern (Figure 7.4A). In the Mass1 and Mass2 groups, there were significantly ($p < 0.05$) higher differences in the period of 1–7 dpi compared with 9 dpi. Between the Mass1 and Mass2 vaccines, there were no significant differences in the viral load at 3, 5, 7 and 9 dpi. The Mass1 vaccinated viral load was significantly ($p < 0.05$) higher than that of Mass2 at 1 dpi.

In the 793B2 group, there was a significantly ($p < 0.05$) higher difference in the period of 1, 5, 7 dpi compared with 2 and 9 dpi (Figure 8.5A). At 1 dpi, the Mass2 group was significantly ($p < 0.05$) lower than the other groups, while at 5 and 7 dpi, the 793B1 showed a significantly lower viral load. Between the 793B vaccines, there were significantly ($p < 0.05$) higher levels in 793B2 at 5 and 7 dpi (Figure 7.4B).

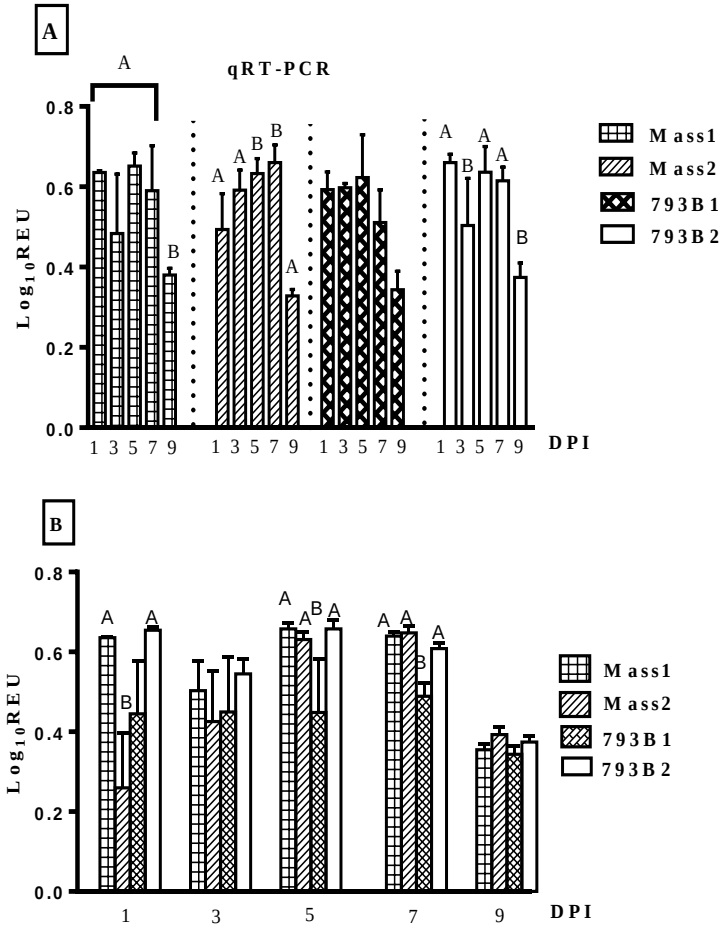

Figure 7.4. Quantification of infectious bronchitis virus (IBV) expressed as log 10REU of RNA in the trachea by real-time RT-PCR following infection in day-old chicks. (A) Within each strain at 1-9 dpi. (B) Within strains at each sampling day. (n = 5; significant differences indicated with different letters, $p < 0.05$). Significant differences were determined using ANOVA.

7.3.6 Local and humoral immunity

7.3.6.1 Measurement of lachrymal antibodies (IgA) by ELISA:

The IgA titres from lachrymal fluid showed no statistical difference among the groups at 7 and 14 dpi. The titre was significantly higher ($p < 0.05$) at 14 dpi compared with 7 dpi in all inoculated groups (Figure 7.5A).

7.3.6.2 mRNA expression of IgA in the lachrymal fluid:

From the three pooled samples, no significant differences were found among the groups. There was up-regulation in the mRNA expression of IgA in the lachrymal fluid at 7 and 14 dpi. The Mass1 vaccine showed the highest expression of IgA compared with the other inoculated groups (Figure 7.5B).

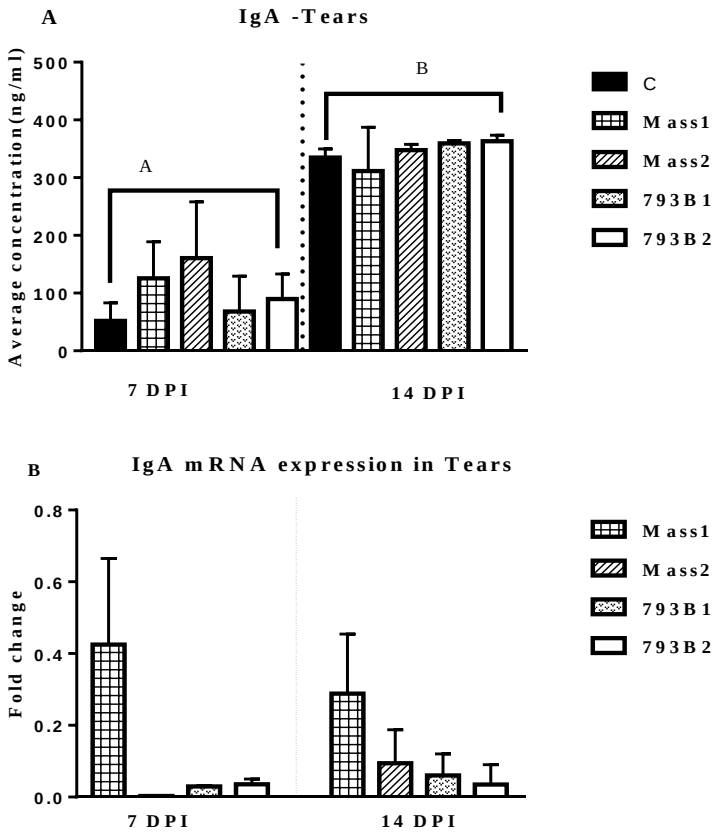

Figure 7.5. Measurement of IgA titre using ELISA and relative mRNA expression from the lachrymal fluid. (A) Mean IgA from tears at 7 and 14 dpi, data expressed as mean (ng/ml). (B) Relative mRNA expression of IgA in the lachrymal fluid, expressed as fold changes divided by the control non-infected group at 7 and 14 dpi. Significant differences are indicated with different letters.

7.3.6.3 IgA expression from tracheal washes:

There was no significant upregulation ($p > 0.05$) in IgA mRNA gene expression for all timepoints (Figure 7.6A).

7.3.6.4 IgA expression from the tracheal tissue:

In the tracheal tissue, there was higher up-regulation in the mRNA of IgA starting from 1–9 dpi in all inoculated groups in comparison to the control group. At 7–9 dpi, the Mass1 and 793B1 inoculated groups were significantly ($p < 0.05$) higher compared with Mass2 and 793B2 (Figure 7.6B).

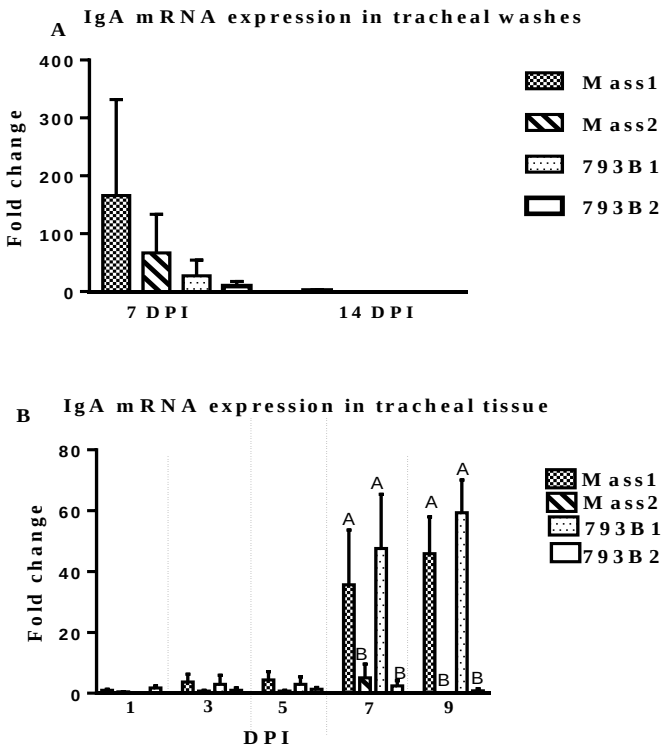

Figure 7.6. Relative mRNA expression of IgA in the tracheal washes and trachea. (A) IgA mRNA expression in the trachea washes at 7 and 14 dpi. (B) IgA mRNA expression in the tracheal tissues at 1–9 dpi. Data is expressed as fold change compared to the control group. Significant differences are indicated with different letters.

7.3.6.5 IgG mRNA expression from lachrymal fluid:

Higher up-regulation of IgG mRNA expression was detected in the Mass1 and Mass2 groups, up to 10 times greater than that of the other vaccinated groups, at 7 dpi. At day 14, mRNA expression of IgG was up-regulated to similar levels in all groups, without any significant differences between them, which was up to 100 times that of the control group (Figure 7.7A).

7.3.6.6 Anti-IBV-specific antibody titre using ELISA (systemic Ab):

There were no significant differences ($p > 0.05$) between the inoculated groups at 7 and 14 dpi for serum IgG (Figure 7.7B).

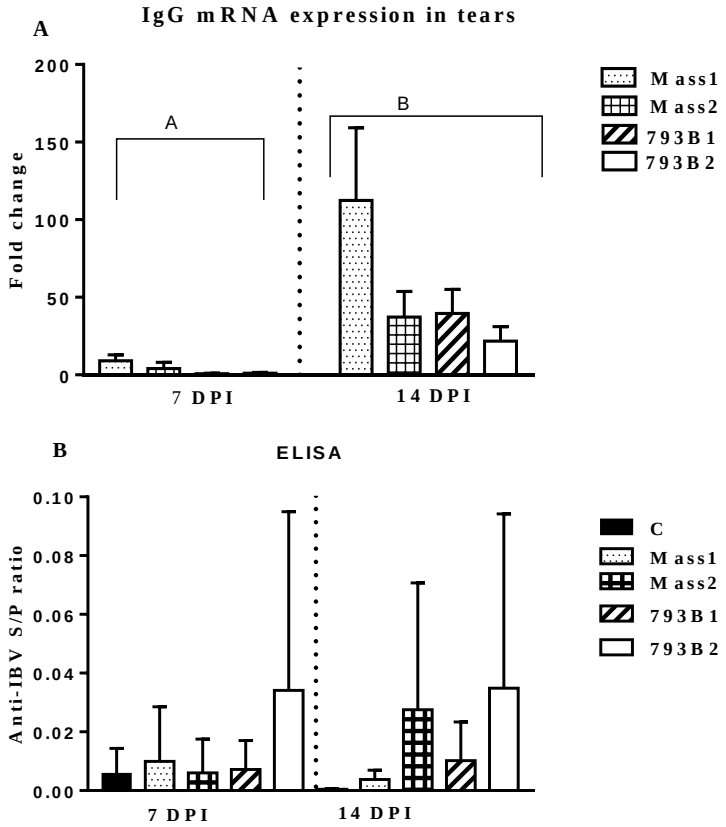

Figure 7.7. Relative mRNA expression of IgG and IgG anti-IBV titre in the lachrymal fluid and serum. (A) mRNA expression of IgG in the lachrymal fluid expressed as fold change divided by the control non-infected group at 7 and 14 dpi. (B) Mean IgG serum level expressed as mean S/P ratio at 7 and 14 dpi.

7.3.6.7 IgG expression from tracheal washes:

Expression of IgG mRNA was up-regulated at 7 and 14 dpi in all inoculated groups. Day 14 showed peak expression levels, which was higher in the Mass2 and 793B2 groups than the other inoculated groups and up to 20 times more than the control group (Figure 7.8A).

7.3.6.8 IgG expression from tracheal tissue:

Expression of IgG mRNA was up-regulated early at 3 dpi in the Mass1 and 793B1 groups compared with the other vaccinated groups. No significant changes were found between the groups or on any day. The highest up-regulation was at 7–9 dpi in allvaccinated groups, with Mass1 having the highest up-regulation (Figure 7.8B).

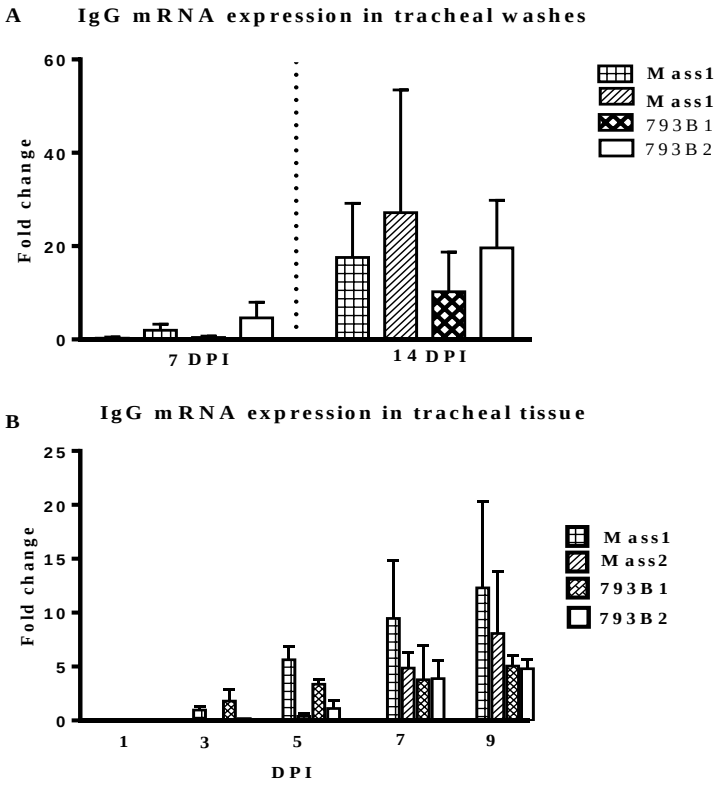

Figure 7.8. Relative mRNA expression of IgG in the trachea and tracheal washes. (A) IgG mRNA expression in the trachea washes at 7 and 14 dpi. (B) IgG mRNA expression in the trachea at 1–9 dpi expressed as fold change divided by the control non-infected group. Different letters indicate significant differences.

7.3.7 Innate immune responses in the trachea

The host innate immune responses were evaluated by assessing the mRNA expressions of IFN-α, IFN-β, MYD88, TLR3 and MDA5 of the tracheal tissues.

7.3.7.1 Relative IFN-α, IFN-β and MYD88 mRNA expression

No significant upregulation ($p < 0.05$) was seen in the mRNA expression of IFN-α at any sampling interval (Figure 7.9A). There was significantly higher up-regulation ($p < 0.05$) in IFN-β mRNA expression at 3 dpi for the 793B1 and 793B2 groups compared with the other inoculated groups (Figure 7.9B). There was up-regulation in the mRNA expression of MYD88 gene in all inoculated groups in comparison with control group. No significant differences were seen among inoculated groups, where 793B2 had the highest expression for all the sampling days (Figure 7.9C).

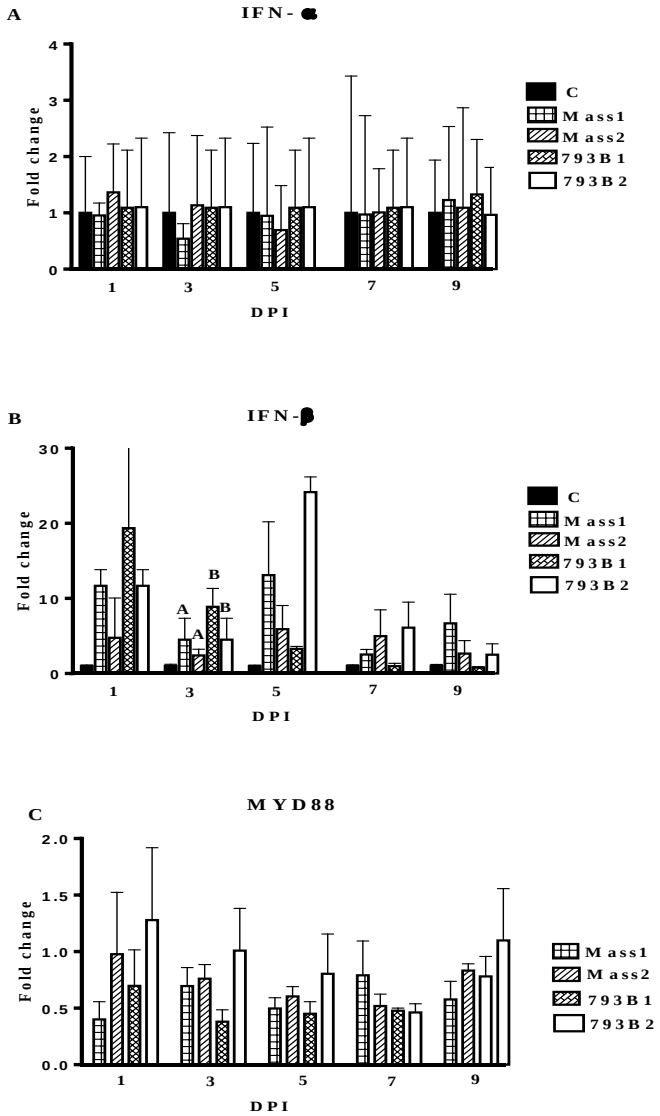

Figure 7.9. Relative expression of innate immune response mRNA genes in tracheal samples experimentally inoculated with IBV vaccines or mock-infected chicks. (A) IFN-α, (B) IFN-β, (C) MYD88; different letters indicate significant differences ($p < 0.05$).

Comment [GK10]: Are sure? That there was no significant differences???? See discussion!

7.3.7.2 TLR3 and MDA5 mRNA expression

There was up-regulation in the mRNA of TLR3 in all inoculated groups at 1–7 dpi, which was significant ($p < 0.05$) at 5 and 7 dpi compared with the control group (Figure 7.10A). The Mass1, Mass2 and 793B2 groups were significantly higher when compared with the 793B1 group at 5 and 7 dpi. For the MDA5 mRNA, there were significant up-regulation ($p < 0.05$) at all sampling days when compared with the control group. Among the vaccinated groups, the 793B1 and 793B2 groups were significantly higher ($p < 0.05$) at 3 and 5 dpi respectively, when compared to the other vaccine groups (Figure 7.10B).

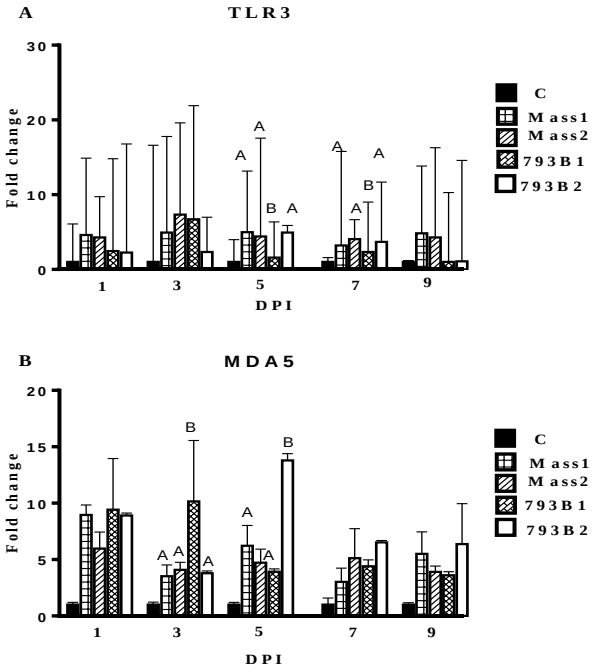

Figure 7.10. Relative expressions of sensing molecules for mRNA genes in tracheal samples after infection. (A) TLR3 mRNA expression and (B) MDA5 mRNA expression; different letters denote significant differences ($p < 0.05$).

7.3.7.3 Proinflammatory cytokine responses in the trachea

There were no significant changes in the levels of IL-1β gene expression at any timepoints, with the exception of the Mass2 group, which was significantly ($p < 0.05$) down-regulated at all sampling days compared with the control and other inoculated groups (Figure 7.11A). At 3 and 5 dpi, significant ($p < 0.05$) down-regulation in the mRNA expression of IL-6 was seen in the Mass2-inoculated group compared with the control and other inoculated groups. At 5 dpi, there was significantly ($p < 0.05$) higher up-regulation in the 793B2-inoculated group compared with the control and other inoculated groups. There was significant down-regulation ($p < 0.05$) at 7 and 9 dpi in the Mass2, 793B1 and 793B2 groups compared with the control non-infected group (Figure 7.11B).

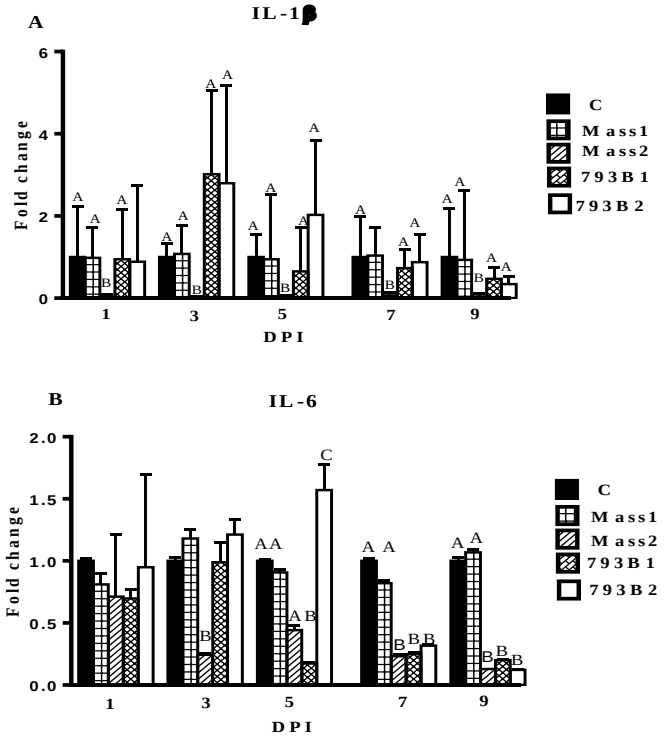

Figure 8.11. Relative expression of proinflammatory mRNA gene expression in tracheal samples from inoculated or mock-infected chicks. (A) IL-1β, (B) IL-6 in tracheal samples; different letters denote significant differences ($p < 0.05$).

7.3.8 Cell-mediated immune responses in the trachea

The mRNA expressions of IFN-γ and CD8+, and changes in the CD4+:CD8+ ratio, together with the immunohistochemistry counts of CD4 and CD8, were evaluated from either the trachea or blood.

7.3.8.1 mRNA expression of type IFN-γ and CD8+

There was over-expression of IFN-γ mRNA in all inoculated groups on all sampling days compared with the control group. At 1–7 dpi, there was significantly ($p < 0.05$) higher up-

regulation in the Mass1, Mass2 and 793B1 groups compared with the 793B2 group. At 7–9 dpi, there was significantly ($p < 0.05$) higher up-regulation in the Mass groups compared with the 793B groups (Figure 7.12A).

There was over-expression of CD8β mRNA in all the inoculated groups on all sampling days compared with the control group. At 1, 5 and 7 dpi, there was significant up-regulation ($p < 0.05$) in the Mass2, 793B1 and 793B2 groups compared with the Mass1 group. At 9 dpi, there was significant up-regulation ($p < 0.05$) in the 793B2 group compared with the Mass1, Mass2 and 793B1 groups (Figure 7.12B).

7.3.8.2 Immunohistochemistry counts of CD4+ and CD8+

There were no significant differences in the CD4+ and CD8+ counts at 7 dpi in all the inoculated groups compared with the control group. However, at 14 dpi, the counts were significantly ($p < 0.05$) higher among the inoculated groups compared with the control group.

At 14 dpi, the average total number of CD4+ cells was significantly ($p < 0.05$) higher in the Mass1, Mass2 and 793B2 groups compared with the control group. In the 793B2 group, the average total number of CD4+ cells was significantly ($p < 0.05$) higher compared with the other inoculated groups. The CD4+ counts were significantly ($p < 0.05$) higher in Mass2 compared with Mass1.

At 14 dpi, the average total number of CD8+ cells was significantly ($p < 0.05$) higher in the Mass1, Mass2 and 793B2 groups compared with the control group. In the 793B2 group, the average number of CD8+ cells was significantly ($p < 0.05$) higher compared with the other inoculated groups. The average number of CD8+ cells was significantly ($p < 0.05$)

higher in the 793B2 group compared with the 793B1 group. No differences were found in the Mass groups (Figure 7.12C and Figure 7.13).

7.3.8.3 Mean CD4+: CD8+ T ratio in blood samples

There were no statistically differences in the CD4+ and CD8+ T ratios in the peripheral blood among the groups. At 14 dpi, all inoculated groups had a higher ratio compared to the control group (Figure 7.12D), whereas at 7 and 14 dpi, the 793B2 group had a non-significant increase of CD4+ and CD8+ in comparison with the other groups.

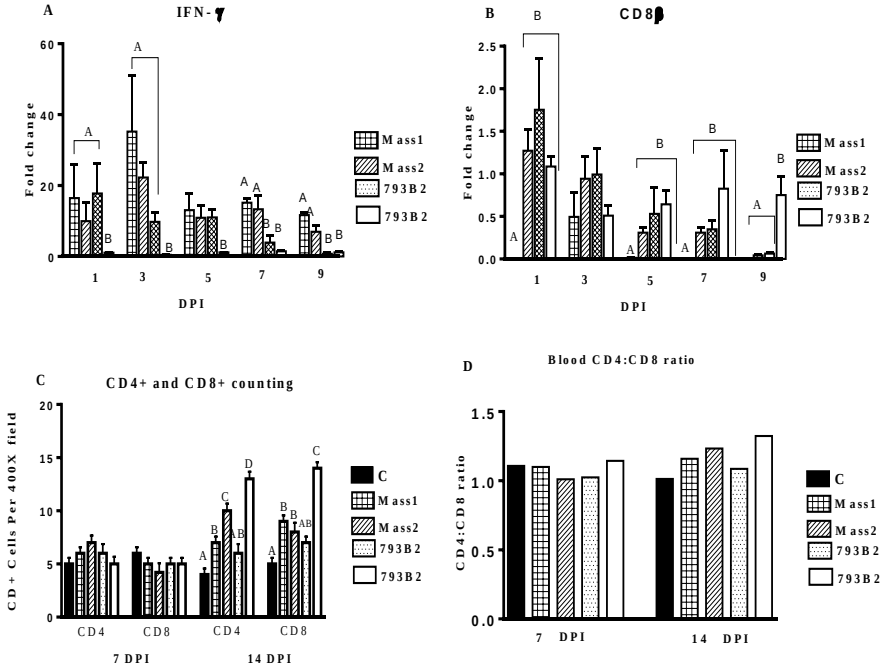

Figure 7.12. Cell-mediated immune responses in the trachea and blood. (A) Relative expression of IFN-γ in the trachea. (B) mRNA expression of CD8β gene in the trachea. (C) Total counting of CD4+and CD8+ cell using immunohistochemistry from the trachea. (D) Mean CD4+: CD8+ T ratio in blood samples using the flow cytometer in different groups at 7 and 14 dpi. Significant differences at different timepoints was determined using by ANOVA (as $p < 0.05$) in comparison with the control group. Different letters denote significant differences ($p < 0.05$).

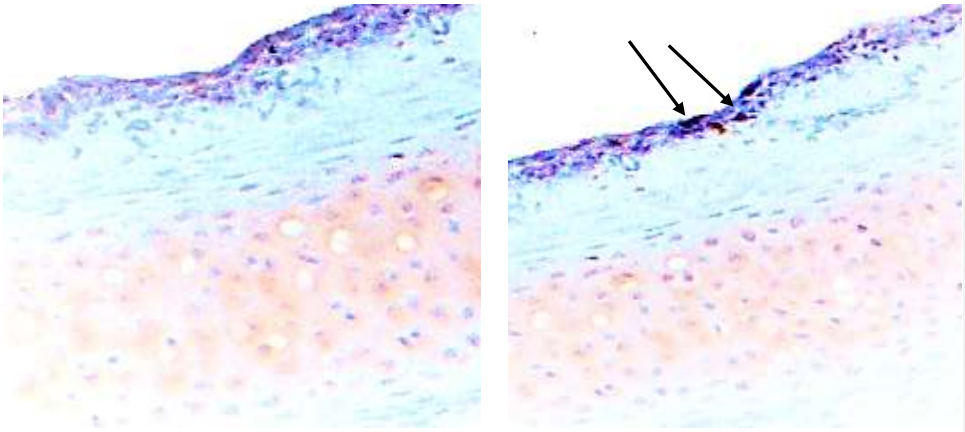

Figure 7.13. Immunohistochemical detection of CD8+ cells in the Mass1 vaccinated group, 7 dpi. Magnification = 400x. Arrows indicate positive cells.

7.4 Discussion

It is well known that IBVs mainly replicate in epithelial cells of the bird's upper respiratory tract, leading a series of processes, including pathogen recognition and robust immune responses, often resulting in tissues damages reflected by pathology and clinical signs. The underlying mechanisms such as host innate immune responses, to date, have not been well investigated. Few reports are available on innate immune responses following either virulent infection (Okino *et al.*, 2017) or vaccine inoculation (Smialek *et al.*, 2017). These publications reported variable results, possibly due to differences in the virulence of viruses used (Chhabra *et al.*, 2018), dosages applied (Okino *et al.*, 2013) or the particular chicken lines used in the studies (Smith *et al.*, 2015). In the current study, the pathobiology and innate immune responses were assessed following oculonasal application of four common but widely used live vaccines (Mass1, Mass2, 793B1, 793B2) in day-old SPF chicks.

In this study, mild respiratory signs were accompanied by mild histopathological lesions in the trachea. Such changes may associated with the virulence levels of these live vaccine viruses (see Chapter 6, TOC). Similar to the current study, the histological changes frequently reported after IBVs infection in SPF chicks such as virulent M41(Chhabra *et al.*, 2018) and 793B(Chhabra 2016).

Tracheal viral load differed, depending on the vaccine strain. Within-period comparisons showed a significantly greater increase in Mass1 and Mass2 at 1–7 dpi compared with 9 dpi. Moreover, 793B2 demonstrated a lower viral load at 3 and 9 dpi compared with the other sampling days. Despite the same inoculation dose, 793B1 infected trachea had a much significant lower viral load compared to other groups in the period of 5–7 dpi. With this, there was also lower mRNA expression of IL-1β at 1–9 dpi and the lowest MDA5 expression at 5 and 9 dpi compared with the levels in the other inoculated groups. This suggests that with greater virus proliferation, there are higher innate immune responses at the molecular level. It is well known that virulent 793B strains are acknowledged for their diverse antigenic structures, pathogenicity levels and tissue tropisms (Cook *et al.*, 1999). In the *in vitro* work, it was explained that 793B1 has a late RCA 50% time point, indicating a lower proliferation rate and less virulence.

In this study, data revealed upregulation in MYD88 gene expression in the period of 1–9 dpi for all the inoculated groups. These results is similar to those of Okino *et al*., (2017), who also found up-regulation in this gene up to 8 dpi; however our vaccine strains induced a lower fold change compared to the virulent strains used by Okino *et al*., (2017). This discrepancy suggests that expression likely associated to IBV virulence. This gene (MYD88) is one of several involved in signal transduction, resulting in the production of

IFN-β (Schat *et al.,* 2014). At the end of activating MYD88, macrophage inflammatory protein(MIP)-1β, IL-12, IL-8, IL-6 and IL-1β become activated by the cytokine cascade, normal T cells expressed and secreted (RANTES) are expressed (He *et al.,* 2016).

A key finding from this study is that up-regulation of IFN-β, TLR3 and MDA5 mRNA expression occurs in the period of 1–9 dpi, with greater expression in the primary stage of inoculation at 1–5 dpi. It has been found that these cytokines and PRRs (TLR3 and MDA5) are activated with either IBV infection or vaccination (Wang *et al.,* 2006; Kameka *et al.,* 2014; Zhang *et al.,* 2017). Such findings support the theory that TLR3 pathways are stimulated by all IBVs, however more so for virulent strains, initiating greater production of IFN-β through TIR-domain-containing adapter-inducing interferon-β (TRIF) (He *et al.,* 2016).

In all the groups, the highest average IgA titre from the lachrymal fluid was observed at 14 dpi, suggesting the delay in humoral immune response is due to B lymphocyte recruitment, which are the main precursor of this antibody isotype. Similar patterns were found for H120 (Okino *et al.,* 2013) and H120+CR88 vaccines (Chhabra *et al.,* 2015a). Lachrymal fluid–specific IgA antibodies to IBV is reported to indicate immune status against challenge in the field (Toro and Fernandez 1994). In this study, lachrymal fluid IgA antibody levels were highest when compared with this immunoglobulins levels tested from tracheal washes and tracheal tissue. This suggests the importance of the Harderian gland (HG) in contributing to local immunity through IgA immunoglobulin production. Oculonasal inoculation potentially results in a robust local immunity present in the HG, originating from the higher IBV exposure, although in the HG the viral load was not investigated in the current study.

Expression of mRNA of IgA from tracheal tissue was significantly higher in the Mass1 and 793B1 groups at 7–9 dpi compared to other inoculated groups, as well as grater expression compared to the other groups from the tracheal washes at 7 dpi, in the Mass1 group. This increase might be associated with resistance factors that are present in White Leghorn lines, such as line C, but not line 151, despite having similar average IgG levels over a certain time period after infection with M41 (Cook *et al.,* 1992). In the tracheal washes, a similar increase of IgA antibody has been reported in broiler chickens vaccinated with either Ma5 or Ma5 combined with 4/91 (Smialek *et al.,* 2017).

Proinflammatory cytokine (IL-1β and IL-6) findings indicated higher up-regulation in IL-1β mRNA expression at 3 dpi in the 793B1 and 793B2 groups, which became almost double that of the Mass and control groups. Conversely, previous work has shown that M41 resulted in greater upregulation in the expression of IL-1β and IL-6, which was connected with higher viral loads, gross lesions and microscopic changes (Okino *et al.,* 2014). High expression of IL-6 has a detrimental effect on lesions (Asif *et al.,* 2007), and both IL-1β and IL-6 are well known to be accompanied by a high viral load and damaged tissue in the trachea and/or kidney (Chhabra *et al.,* 2015b). This could be the consequence of reduced up-regulation of MYD88 in the current study. The effect of the TLR7 pathway (which incorporates MYD88), should be considered in future work. It is likely that the TLR7 pathway is mostly activated by virulent IBVs, which then mediates the IL-6 and IL-1β proinflammatory cytokines, resulting tissue damage.

Significant differences were found within all inoculated group tracheas at 14 dpi for both CD4+ and CD8+ counts using immunohistochemistry. Data showed that 793B2 had the highest average in CD4+ and CD8+ at 14 dpi compared with other inoculated groups.

Apart from Mass1, the recruiting of CD8+ was greater than that of CD4+, which is a similar finding to what has been previously reported after 793B infection in SPF and broiler chickens (Raj and Jones 1996a). These significant differences have been reported not only for single IBV vaccine applications, but also for combinations of vaccines from other viral species, such as Newcastle Disease virus and avian metapneumovirus (Awad *et al.,* 2015). In the current study, the peripheral blood CD4+:CD8+ ratio peaked in all vaccinated groups at 14 dpi. These results is comparable to those outlined previously (Kjærup *et al.,* 2014; Chhabra *et al.,* 2015b). The increase in this ratio indicates the status of immune protection against viral infection (Dalgaard *et al.,* 2010). It may be debated which IBV strains induce a higher host immunological response and which cells are more imperative during viral invasion and clearance, as an increase in CD4 cells was found in SPF chicks after infection with VI648 in (Janse *et al.,* 1994), but higher CD8 cell components were reported after 793B IBV infection (Raj and Jones 1996a).

In the 1–7 dpi period, there was up-regulation of IFN-γ mRNA in all vaccinated groups. However, the magnitude of expression was variable, with little expression in 793B2. This type II IFN is predominantly produced by NK cells, NK T cells, dendritic cells and Th1 CD4+ and CD8+ effector cytotoxic T lymphocytes (Vervelde *et al.,* 2013). Previous work outlines that this IFN is up-regulated after either infection or vaccination with IBV (Okino *et al.,* 2013; Okino *et al.,* 2014; Okino *et al.,* 2017). IFN-γ and Granzyme homolog A are well documented to combine with the action of NK cells, which initiate the innate immunological response during IBV M41 challenge (Vervelde *et al.,* 2013). Data in the current study showed that there is significantly higher up-regulation in type II IFN at 3–9 dpi in the Mass group when compared with the 793B group; the causes of such differences are unclear.

The CD8β mRNA gene expression was up-regulated in all groups, with reduced expression in Mass1 at 1–7 days compared to the other vaccines. This cell-mediated immune (CMI) gene is considered a marker of the effector activity of CD8 T cells induced by IBV vaccination in tracheal tissues. Similar up-regulation was reported at 5–8 dpi in SPF chicks with Brazilian IBV isolates (Okino *et al.*, 2017). It has reported that, after IBV infection in the trachea, the adaptive immunity will be established against infection in 3–7 days; moreover, an influx of cells, such as CD8+ and CD4+ lymphocytes, will occur, and the local CMI response will be initiated (Kotani *et al.*, 2000; Chhabra *et al.*, 2015b). At the molecular level may be such differences arose due to changes in the non-structural and structural proteins of IBV strain, which interacted during viral invasion and initiated the immune response, as well as different pathways followed by various strains (Zhong *et al.*, 2012).

In the previous chapter, 793B2 reached EP50% in the shortest timeframe, suggesting this strain causes a higher level of virulence amongst the vaccines investigated. In addition, the 793B2-inoculated group showed higher cell-mediated immune responses, represented by higher CD8β gene expression in the period of 5–9 dpi, higher CD4 and CD8 counts at 14 dpi and higher CD4:CD8 ratios at 7 and 14 dpi. The cause of such response is not known, but it may be connected to virulence, and further studies could look to interpret these differences.

Data gathered in this study indicated that the prominent components of resistance against IBV challenge such as CD8+, IFN-β, IFN-γ gamma, IgG and IgA. All strains used in the current study were immunogenic in SPF chicks. Furthermore, the 793B2 group induced the high level of CD8 cells, systemic antibodies and MYD88 and the highest IgA fold change,

which could be associated to the virulence of this strain. Further investigation is needed using bivalent vaccination regimes rather than the monovalent one used in the present study.

Chapter 8: General discussion and future work

The causative agent of infectious bronchitis (IB) is avian infectious bronchitis virus (IBV), which is a positive sense RNA virus that infects the respiratory tract, kidneys and digestive organs depending on the tropism of respective IBV strains. The virus S1 gene is characterized by high genetic variability and possesses three hypervariable regions (HVRs) that are responsible for the induction of neutralizing antibodies, which is generally serotype specific (Moore *et al.,* 1997). A number of factors promotes emergence of new genotypes of IBVs in different parts of the world and further evolution of these strains plays the central role in the development of new variants (Jackwood 2012). Point mutations, insertions, deletions, and recombination among strains have influenced the emergence of new variant (Hewson *et al.,* 2014). Therefore, lack of cross-protection between the immunity produced by currently available vaccines and emerging variants have been widely reported in recent years. This has led to substantial economic losses due to IBV, where after NDV and AIV, IBV is considered as the most important threat to chicken health, production and welfare worldwide.

An RT-PCR scheme was developed to amplify and sequence the full S1 gene of classical (M41) and variant (D274, 793B, IS/885/00, IS/1494/06, Q1 and QX) (Chapter 3). The sensitivity of this scheme was evaluated after FTA cards were inoculated with four IBV viruses and stored at three temperatures (4, 40, and 22 $^{\circ}$C). Partial S1 sequences exhibited a higher average nucleotide homology percentage (79%; 352bp) when compared to full S1 sequences (77%; 1,756bp), suggesting that the full S1 protocol has shown a greater efficacy for strain identification. The work in Chapter 3 showed the following: -

a. The greater sensitivity seen with partial S1 amplification may be in part due to the considerable difference in amplicon length. Data has shown that the partial

assay was able to detect IBV RNA inoculated onto FTA cards up to 21 dpi for all storage conditions (including 40°C). However, this was not possible for the full S1 gene, showing that the sensitivity for amplifying the larger region (full S1) is affected by both incubation time and temperature. This finding is in agreement with a recent study which had difficulty in amplifying a product greater than 900 bp, despite detecting a 290 bp product after six months of storage (Sakai *et al.*, 2015).

b. Usual practice of IBV diagnosis in local or international laboratories is through the collection of oropharyngeal swabs, tissue samples collected at necropsy or IBV-enriched allantoic fluids embedded onto FTA cards. Samples are then stored, and/or transported at different temperatures for variable lengths of time. The study was designed to mimic conditions that field samples may be subjected to prior to extraction of RNA and our full S1 scheme. It appears that storage of FTA cards at 40 °C must be avoided and least favoured for detection of IBVs. For samples on FTA cards, detection of partial rather than full S1 is preferred, as for the latter protocol, it appears higher RNA quantities are needed.

Future work to improve the sensitivity of the assay would improve the usefulness for field sample testing. Currently, the full S1 sequencing scheme appears to be suited for samples enriched in growth medium (e.g. eggs) rather than those obtained directly from the field (e.g. tissues, swabs and impression smears).

As stated in the previous chapters, despite the wider detection of IBV Q1 in different continents of the world (except Central and North America and Australasian counties), the

pathogenicity of this virus has not been probably studied. Also, Chabbra et al. (2018) reported on the virulence, pathogenicity and early immune responses to IBV M41, QX and 885 but not on Q1. As such, in Chapters 4 and 5, the immunopathogenesis of Q1 IBV was investigated in SPF (Chapter 4) and two broiler lines, Line-A and Line-B, with differing growth rates (Chapter 5). The relative importance of the various resistance factors, such as innate immune responses, which play a key part in IBV pathogenicity, is not yet fully understood. Research onto the three types of chickens provided scientific findings for the following conclusions: -

a. The Q1 variant isolate used in this study was pathogenic to SPF and both breeds of broiler chicks. It appears that the Q1 has higher tropism to the trachea rather than the kidneys.
b. In both SPF and broiler chickens, the Q1 infection can cause significant body weight reduction. Further studies could be helpful in terms of including more breeds to assess this point.
c. The Q1 was isolated from the proventriculus from all infected groups was successful but attempts to show the replication of the virus in this tissue by IHC was not successful. Probably, the investigation should include use of other monoclonal antibodies, different dilutions of the antisera, green fluorescent protein (GFP) labelled IBV, more frequent sampling intervals, as truly demonstrating local replication of IBV in the proventriculus would add new dimension on IBV pathogenesis.
d. Comparison between the broiler lines (fast *versus* slow growers), it was demonstrated that the fast-growing birds are more susceptible to IBV Q1, where the virus able to persist throughout the experimental duration in various tissues.

This interesting and important finding likely to raise welfare questions on the use of fast growing broiler breeds in the industry. In fact, higher susceptibility for fast growing broiler breeds to *Salmonella* infection (van Hemert *et al.*, 2006), *Campylobacter jejuni* (Williams *et al.*, 2013; Humphrey *et al.*, 2015) have been reported before.

e. For the sensing genes, IFNs and pro-inflammatory cytokines, in SPF infected trachea, the next genes were up regulated in all sampling days TLR3 and TNF-α. In the SPF infected kidney, the next genes were up regulated in all sampling days IFNα, IFNβ, TLR3, MDA5 and LITAF. IFN-α was up regulated only in Line-A but IFN-β was up regulated in both lines. For TLR3, an up-regulation was seen in Line-A up to 7 dpi and for all sampling days in Line-B. MDA5 was up-regulated in Line-A and down-regulated in Line-B at 1 dpi. In the kidneys, for Line-A birds, IFN-α and IFN-β were up-regulated at 1 and 1-3 dpi respectively. There was up-regulation in TLR3 in Line-B throughout the study period but not for Line-A. MDA5 was up-regulated in both lines at 7 and 9 dpi. These mentioned host genes are clear from our data that have a role on the course of infection and then influenced sings or gross and histological alteration extent and then the pathogenicity degree.

In Chapter 6, the aim was to provide further information about the *in vitro* pathogenicity and genetic characteristics of 11 IBV strains (virulent and vaccine strains). Comparisons were made between eight commonly used vaccines and three virulent strains belonging to common serotypes worldwide 793B, Massachusetts and QX. During the study, we utilised trachea organ cultures (TOCs), a commonly described method of assessing the *in vitro*

pathogenicity of IBV on respiratory tract ciliated epithelia. The results demonstrated the following:-

a. Confirmed that there are evident differences between the pathogenic effect of strains in the same serotype and some vaccines can show cilia degradation comparable with that of the virulent strain.

b. The qRT-PCR analysis, which was limited to the TOC media in this study, showed a significant decrease of the viral load in the 24- and 72-h post-infection samples, potentially due to the movement of viral particles from the media into the cells. To explore this hypothesis, further studies focussing on molecular biology analysis on tracheal rings would be necessary to expand the genetic difference evaluation. In addition, cytopathic characteristics could be investigated alongside expanding the study to a larger number of serotypes and extending the interval of TOC incubation. Despite similar ciliostasis effects seen in certain vaccine strains, a greater number of apoptotic cells were found in groups infected with virulent strains. Our conclusion is that linking the MDA5, and IFN-β with the 50% time-point and apoptosis with pathogenicity was associated with greater expression and increased pathogenicity. This study demonstrated the usefulness of using TOCs for experiments investigating pathogenesis, host invasion and genetic variation, reducing the need for *in vivo* trials.

The Chapter 6 provided scientific evidences that pathogenicity and early immune responses between various live vaccines that are available in the market could be

differentiated using an *in-vitro* system. Such infectious model is useful, as use of live animals can be reduced or avoided, and a large number of live IBV vaccines could be screened simultaneously. However, it was important to validate the results on the Chapter 6, at least few of the vaccines in *in-vivo*, to assure that the differentiation can be presented in birds. For this, work in Chapter 7 was undertaken, basically investigating the pathobiology and host immune responses following administration of single live vaccines in day-old SPF chicks. To avoid, excess usage of birds, only two Mass (Mass1, Mass2) and two 793B (793B1, 793B2), vaccines were used. Following findings were derived from the Chapter 7:-

a. From the study data, pathogenicity assessment indicated that, the 793B2 was the most virulence strain among the other vaccinal strains.
b. All vaccines induced local, cellular and innate immune responses. Cloacal swabs and RT-PCR were positive at 1 dpi for 793B1 and 793B2, but not for Mass1 and Mass2. This indicated that the 793B virus induced viraemia in a short time (less than 24 h) and spread to the digestive or kidneys, or possibly the 793B virus has tropism for replication in the intestine.
c. In the lachrymal fluid, there was a higher IgA titre at 14 dpi compared with 7 dpi in all the groups. Levels of IgA antibodies in lachrymal fluid have been associated with IBV infection or vaccination (Gelb *et al.,* 1998; Chhabra 2016; Okino *et al.,* 2017).
d. IgA mRNA expression in tracheal washes indicated higher expression at 7 dpi in the H120-vaccinated group when compared with the other vaccinated groups, and little expression at 14 dpi. This is the first report measuring the expression of IgA in tracheal washes, tears and tracheal tissues individually. Others

working on whole IgA in tracheal washes, reported an increase in the titre following either vaccination or virulent virus infection (Davelaar *et al.*, 1982; van Ginkel *et al.*, 2008). This highlights the importance of this isotype in immune responses and protection against challenges.

e. The host innate immune mRNA gene expressions were elevated for IFN-β, MDA5, TLR3, IL-1β and IL-6 after inoculation. Overall, each vaccine induced a protective level of innate responses, which would help during field homologous challenge. The CMI genes tested in the current study outlined that upregulation of IFN- γ mRNA expression occurred early, at 1 dpi, in all the vaccinated groups. This may suggest an increased importance of this gene in acting as an antiviral agent, as previous work also showed that IFN-γ is the main immunological component of cells like dendritic cells, NK T cells, CD8+ effector cytotoxic cells and Th1 CD4+ T lymphocytes (Vervelde *et al.*, 2013).

f. The data indicated that the 793B2 inoculated group showed higher cell-mediated immune responses, represented by higher CD8β gene expression in the period of 5–9 dpi, increased CD4+ and CD8+ counts at 14 dpi and higher CD4:CD8 ratios at 7 and 14 dpi. This may be considered a promising aspect of these components of host innate, humoral and cellular responses, which could be crucial in protection when homologous challenges occur.

Overall, study findings have significant implications for the understanding of how host and virus interactions at the molecular level represented by innate immune responses. The present study provides the first comprehensive assessment of immunopathogensis of virulent IBV (Q1) and vaccinal viruses.

Study limitations

- In this study, storage of FTA at temperatures below 0 was not investigated. It would have been interesting to shed some answers on this aspect, as temperatures below 0 likely to preserve the viability of RNA. This may allow better detection of IBV, particularly for the full-S1 sequencing.
- The Q1 immunopathogenesis in SPF chicks; current study is not in line with the field conditions, as exacerbations by various adverse environments and other factors such as age and MDA level were not assessed.

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Appendix I (Reagents and protocols)

Stock solution D

Guanidine thiocyanate [64]	100.00 g
0.75M sodium citrate [40]	7.04 ml
10% lauryl sarcosine [40]	10.50 ml
Sterile distilled water	7.04 ml

Kept for one month at 4^{o}C

Working solution D

Stock solution D (see sol D)	25 ml
Mercaptoethanol[40]	180 µl

TOC culture medium and supplements

10x Minimum Essential Medium-Eagles [65]	100 ml
7.5% sodium bicarbonate [40]	20 ml
Sterile distilled water	900 ml
Penicillin-streptomycin solution	4 ml

Stock antibiotic solution

Crystapen[66]	600 mg
Streptomycin sulphate BP [67]	1.0 g

[64] Sigma-Aldrich, UK

[65] Life Technology, UK

[66] Glaxo, Greenford, UK

Sterile distilled water 4 ml

4ml of this stock solution was added to 1000 ml of medium for a 1x solution. For preparing 10x antibiotic medium, 4ml was added to 100ml of medium.

Phosphate buffered saline (PBS)

NaCl 85.00 g

Di-sodium hydrogen orthophosphate (Na_2HPO_4) 10.70 g

Sodium dihydrogen orthophosphate ($NaH_2PO4.2H_2O$) 3.90 g

Made up to 1 litre with sterile distilled water

2M sodium acetate

Glacial acetic acid[68] 19.00 ml

Sodium acetate[69] 5.46 g

Made up to 200 ml with sterile distilled water

0.75M Sodium citrate pH7.0

Citric Acid Monohydrate 31.52 g

NaOH pellets 16.8 g

Sterile distilled water 200 ml

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[67] Evans Medical, Greenford, UK

[68] VWR International Ltd

[69] Sigma-Aldrich, UK

Resuspending (RS) water

RNasin, RNAse inhibitor [70]	2.5 µl
Dithiothreitol (DTT) [71]	5 µl
Ultra-pure water [47]	92.5 µl

Reconstitute stock primers according to delivery details, Dilute stock oligos 1:10 to give working solutions

IBV Oligos[72]

Working Oligo	**Stock Oligo**		**Sigma Water**
SX4-	10µl		90 µl
	A	**B**	
SX1+	10 µl	10 µl	80 µl
SX3+	10 µl	10 µl	80 µl
SX2-	10 µl	10 µl	80 µl

10mM dNTP's Working Solution

dATP, dCTP, dGTP, dTTP at 100mM [51]

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[70] Promega, Madison,USA

[71] Invitrogen, Paisley, Scotland

[72] Invitrogen, Paisley, Scotland

Take 20µl of each dNTP, total volume 80µl

Add 120µl of sigma water to give 200µl of 10mM dNTP's

Nested PCR 1 protocol

PCR Supermix [73]	19 µl	
+ Oligo (IBV SX1+) (aMPV G1+) (MSF-)		0.5 µl
- Oligo (IBV SX 2-) (aMPV G6-) (MSF+)		0.5 µl

Nested PCR 2 protocol

PCR Supermix	23.5 µl	
+ Oligo (IBV SX3+) (aMPV G8-A)		0.5 µl
- Oligo (IBV SX 4-) (aMPV G9+B)		0.5 µl
- Oligo (aMPV G5-0 (aMPV)		0.5 µl

Loading Buffer

Ficol	3 g
1XTBE	20 ml

10x Tris-borate-EDTA (TBE)[74]

This was purchased at 10X concentration and diluted to1X concentration when required.

Gel preparation

1.5% agarose [53] 0.58 g

[73] Invitrogen, Paisley, Scotland

[74] Sigma-Aldrich, UK

TBE 35 ml

cDNA synthesis reaction mixture I[75]

Random hexamers 1µl

10mM dNTP mix 1µl diethyl-pyrocarbonate (DEPC) treated water (up to) 10µl

cDNA synthesis mix[76]

10X RT buffer 2µl

25 mM $MgCl_2$ 4µl

0.1 M DTT 2µl RNasrOUT (40U/µl)

1µl Superscript III (200 U/µl) 1µl

Quantitative reverse transcription PCR (qRT-PCR) reaction mixture

2x Rotor Gene probe[77]	12.5 µl
IBVRT1	2 µl

[75] Invitrogen, Paisley, Scotland

[76] Invitrogen, Paisley, Scotland

[77] Qiagen, UK

IBVRT2	2 µl
IBVRT3 TaqMan® probe	0.5 µl
Rotor-Gene RT Mix[78]	0.25 µl
RNase-free water	5.75 µl

10x Tris-borate-EDTA (TBE)[79]

This was purchased at 10X concentration and diluted to1X concentration when required.

Oligos [80]for RT-PCR

Oligos received were reconstituted as per manufacturer's instructions. The stock

oligos were diluted to 1:10 to make working solutions

RNA extraction protocol using the Qiagen RNeasy Plus kit

Weigh out no more than 30mg of tissue in a 2ml Eppendorf and cut into small pieces using sterile scissors

1. Make up Mercaptoethanol (ME) + RLT Buffer solution
   A. 10µl ME into 1 ml RLT
   B. 100µl ME into 10ml RLT

[78] Qiagen, UK

[79] Sigma-Aldrich, UK

[80] Invitrogen, Paisley, Scotland

C. 500µl ME into 50ml RLT

2. Add 600µl ME+RLT to each 2ml Eppendorf and add a sterile magnetic bead
3. Lyse the tissue for 10 min (trachea or proventriculus) or 8 min (kidney)
4. Centrifuge for 3 min at 13,000rpm
5. Carefully remove the supernatant (around 550µl) and add to a gDNA eliminator spin column (provided in the kit)
6. Centrifuge for 30 sec at 8000g/10,000rpm, discard the spin column and save the flow through in the collection tube. NB make sure that no liquid remains on the column, if necessary repeat the spin
7. Add equal volume of 70% ethanol (e.g. 550µl ethanol into 550µl sample) and mix using the pipette. For max RNA yield from liver use 50% ethanol
8. Transfer 700µl into an RNeasy spin column (provided), centrifuge for 15 sec at 8000g/10,000rpm and discard the flow through.
   A. Repeat this step for any remaining sample.
   B. Reuse the collection tube in next step
9. Add 700µl RW1 buffer and centrifuge for 15 sec at 8000g/10,000rpm then discard the flow through
   A. Reuse the collection tube in next step
10. Add 500µl RPE buffer and centrifuge for 15 sec at 8000g/10,000rpm then discard the flow through
    A. Reuse the collection tube in next step
    B. RPE buffer is supplied as a concentrate – ensure the correct volume of 100% ethanol is added (indicated on the bottle) before using
11. Add 500µl RPE buffer and centrifuge for 2 min at 8000g/10,000rpm then discard the flow through and collection tube
12. Optional: Place RNeasy spin column in new 2ml collection tube and centrifuge for 1 min at13,000rpm
13. Place the spin column in a new 1.5ml collection tube (provided), add 30µl RNase-free water and centrifuge for 1 min at 8000g/10,000rpm to elute the RNA
    A. Repeat this step by adding the flow through back into the collection tube
    B. This ensures a higher yield of RNA in the final
    C.

DNA Extraction using Qiagen Q1A DNA Mini Kit

1. Heat water bath to 56^{o}C and heat lock to 70^{o}C
2. Take 1ml of Solution D (After leaving at -20^{o}C with cut up FTA card for at least 2h) for Virus or Mycoplasma
3. Place in Screw capped eppendorf
4. Centrifuge @13,000g/15min
5. Remove supernatant and discard (you need the pellet)
6. Invert on blue paper to dry
7. To each tube add 180µl of ALT buffer and 20µl pK (proteinkinase)
8. Incubate @ 56^{o}C /15min, vortexing occasionally
9. Add 200µl AL buffer to each tube
10. Votex/15secs
11. Incubate @70^{o}C/10min
12. Add 210µl 100% ethanol to each tube
13. Vortex /15secs
14. Transfer to Q1Aamp spin column
15. Centrifuge @10,000g (11,300rpm)/1min
16. Discard flow through
17. Using new collection tube
18. Add 500µl AW1 buffer
19. Centrifuge @10,000g (11,300rpm)/1min
20. Discard flow through
21. Using new collection tube
22. Add 500µl AW2 buffer
23. Centrifuge @10,000g (11,300rpm)/3min
24. Discard flow through
25. Centrifuge @10,000g (11,300rpm)/3min
26. Discard flow through
27. Place spin column in clip top eppendorf
28. Add 150µl Sigma water, stand @room temperature for 1min
29. Centrifuge @10,000g (11,300rpm)/1min
30. This is the DNA extract, store @ 4^{o}C over night or longer @ -20^{o}C

QlAamp viral RNA extraction

Equilibrate samples and buffers to RT

Addition of carrier RNA to buffer AVL

1. Add 310 µl Buffer AVE to the tube containing 310 µg lyophilized carrier RNA
2. Dissolve the carrier RNA completely
3. If extracting 55 samples add the 310 µl reconstituted carrier RNA to 31ml Buffer AVL
4. This is stable in the fridge for 48h. Look out for precipitation. To dissolve any precipitate, heat at 80^{o}C for no more than 5 min (no more than 6 times)
5. If extracting smaller numbers of RNA see table in booklet with kit for amounts and storage of reconstituted carrier RNA

Protocol

1. Add 560 µl of prepared buffer AVL containing reconstituted carrier RNA into 1.5 ml eppendorf tubes
2. Add 140 µl of sample
3. Vortex for 15sec
4. Incubate at RT for 10min
5. Pulse centrifuge
6. Add 560 µl of 100% ethanol
7. Vortex for 15sec
8. Pulse centrifuge
9. Label spin columns
10. Carefully remove 630µl from tube (step 6) into spin column
11. Centrifuge at 8000 rpm for 1minute, discard flow through
12. Place spin column in clean collection tube and repeat steps 10 and 11
13. Place spin column in clean collection tube add 500 µl of buffer AW1
14. Centrifuge at 8000 rpm for 1min, discard flow through
15. Place spin column in clean collection tube add 500 µl of buffer AW2
16. Centrifuge at 13,000 rpm for 3minutes, discard flow through

17. Place spin column in clean 1.5 ml eppendorf tube add 60 µl of sigma water
18. Incubate at RT for 1 minute
19. Centrifuge at 8000 rpm for 1min, discard spin column and save flow through (RNA)
20. Store RNA at -20^{o}C

Cell Preparation for Flow Cytometry

1. Heparin Preparation (Sigma H4784-250 mg @ 180 usp/mg)
    a. Need to use it @10 usp per 1ml blood
    b. Take 10 mg heparin into 1 ml Normal sterile saline (NSS)
    c. Equals 1800 usp/1000µl NSS, equals 1.8 usp/1µl, equals 9.9 usp/5.5 µl
2. Therefore add 5.5 µl of heparin solution per 1ml of blood to each collection tube
3. Further dilute sample 1:1 with saline or RPMI 1640 medium
4. Add 0.5 ml of histoplaque to a 1.5 ml Eppendorf tube
5. Overlay 1 ml of blood mixture on top of the histoplaque
6. Centrifuge @8000 g or 10000 rpm @ Room Temp for 90 secs
7. Remove the formed buffy layer (middle layer) very carefully by going through the top layer, using a pipette and steady hand
8. Wash gently two to three times with saline or RPMI 1640 medium, spinning @ 500g or 1200 rpm for 5 mins
9. Adjust the cell count to 1 x10^{7} cells per ml
   (1ml at 1:1 suspension = more than 1 million cells (Ross 308))

Immunostaining for flow cytometry

1. Re-suspend cells in 0.5 % BSA in PBS(Blocking solution) final volume 500 µl
2. Aliquot into 100 µl
3. incubate @ room temp for 15 mins
4. Spin down cell suspension @ 1200 for 5 mins
5. Prepare the antibodies @ 0.5µg/10^{6} cells (Normally obtain 10^{6} cells after histoplaque separation thus, in 100 µl have approx. 2 x 10^{5} cells, then you need to add 0.1µg of antibody which is 0.2 µl in 100 µl of cell suspension

6. Incubate cells with antibodies conjugated to FITC in blocking solution in the dark for 30 mins
7. Spin down cells @1200 rpm for 5 mins and wash twice with PBS
8. Finally re-suspend cells in 2% paraformaldyde (final volume 500µl)
9. Stained cells are detected by flow cytometry to determine the % of CD4 and CD8 lymphocytes, using unstained cells as a negative control to adjust the threshold

Protocol for Flow Cytometer Accuri C6

1. Ideally 10^6 cells/ml per sample
2. Ideally 106 cells/ml per sample
3. Samples can be as small as 25 µl, but it is best to use 50-100 µl (50000 counts is good enough)
4. Always include an unstained control
5. FITC-B1(FL1) channel

Procedure

1. Instrument will need 15-30 mins to warm up
2. Switch on by touching screen
3. Check all bottles are blue
4. Instrument has three lasers:
    a. 405 (Violet, VI)
    b. 488 (Blue, B1-B4)
    c. 635 (Violet, R1-R2)
5. Can work on Eppendorfs
6. Create a user folder
7. Switch symbol – acquisition
8. Follow instructions for priming (Cleaning/washing instrument takes about 5 mins)
9. Green lights – Ready

Calibration

1. Go to ‘’New Workspace’’ or select a previous workspace
2. Go to collect
3. Select an empty well

4. Check settings:
    a. Speed Low is best
    b. Limits – 10000- to 50000 events
    c. Threshold (400000 ??)
    d. Uptake volume
    e. Sample volume
    f. Mixing
5. Select the channels you want to work with, always select FSC and your stain, in this case FITC
6. Press Run
7. Adjust plot specifications
8. Use ‘’Backflush’’ between samples
9. Always wash instrument after use
10. Switch off (will take 7 mins)

H&E Frozen Sections

1. 95% Ethanol - dip until clear
2. Running tap water - dip until clear
3. Distilled water - dip until clear
4. Haematoxylin 30 seconds
5. Running tap water - 30 seconds
6. 95% Ethanol - dip 3-4 times
7. Eosin Y - dip 1-2 times
8. 2x 95% ethanol- dip 5-10 times
9. 3x 100% Ethanol - dip 5-10 times 10. 2x Xylene - dip 5-10 times
11. Coverslip with DPX

Virus isolation in eggs

Centrifuge ground up tissue samples

First passage (10)

1. Use 3 eggs per sample plus 3 control eggs
2. Inoculate eggs with 200 µl of sample
3. Incubate for 48h
4. Candle eggs after 24h and discard any dead embryos
5. After 48h candle eggs, note any dead's then put all eggs in the cold room for 24h/overnight
6. Collect the allantoic fluid (AF) and centrifuge (Beware of any contaminated AF)
7. Then carefully remove the embryos to examine for typical signs of IBV infection

Second passage (20)

1. Use 3 eggs per sample plus 3 control eggs
2. Inoculum is 10 allantoic fluid
3. Then as above from No 3

Third passage (30)

1. 3 eggs per sample plus controls
2. Inoculum is 20 allantoic fluid
3. Incubate for 5 days
4. Candle eggs after 24h and discard any dead embryos
5. After 3 dpi candle eggs, note any dead's then put in the cold room
6. After 5 dpi candle eggs, note any dead's then put all eggs in the cold room for 24h/overnight
7. Collect the allantoic fluid (AF) and centrifuge (Beware of any contaminated AF)
8. Then carefully remove the embryos to examine for typical signs of IBV infection

After collecting the AF from the 30, extract the RNA and carry out RT-PCR

If positive for IBV then send the PCR product for sequencing

Protocol for IBV IHC from, frozen sections

1. Fix in acetone 10 min room temperature
2. Air dry room temperature 10 min
3. Draw around sections with FAT pen
4. Wash slides 3x2min TBS
5. Peroxidase Block - apply 100µ1 (or enough to cover section) DAKO REAL 10mins, room temperature
6. 3x2min TBS wash
7. Apply 100µ1 (or enough to cover section) of primary antibody mouse monoclonal anti chicken c IBV (Prionics) dilution? (1: 1000)µsed in formalin fixed paraffin embedded IHC).

4'C overnight Use humidified chamber to prevent slides drying out.

8. 3x2min TBS wash ?
9. Apply 3 drops of DAKO ENVISION +HRP (a mouse), 30mins,.r,o\om temperature
10. 3x2min TBS wash
11. DABW5mins (use Impress Kit)
12 .5min TBS wash
13. 5min dH20 wash
14. Counterstain Haematoxylin 1 min
15. "Blue" running tap water 5min
16. Dehydrate xlmin 96% Ethanol,2x3min 100% Ethanol,Xylene
17. Coverslip sections with DPX

Appendix I *Reagents and protocols*