Immunopathogenesis of infectious bronchitis virus in chickens: the role of head-associated lymphoid and respiratory tissues

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Abstract

Infectious bronchitis virus (IBV) primarily replicates in the epithelial tissues of the respiratory tract, predominantly trachea. Little information is available on IBV replication and immune responses in head-associated lymphoid tissues (HALT) – Harderian Gland (HG) and choanal cleft, and respiratory (turbinate) tissues. To investigate the role of these tissues in comparison to trachea, 21-days of age commercial broiler chickens were challenged with IBV M41. Lachrymal fluid anti-IBV IgA levels were elevated at 4–5 days post-challenge (dpc). At 5 dpc, the viral load in the turbinate and choanal cleft was higher than those found in the HG, pharyngeal and tracheal tissues. Immunohistochemistry (IHC) confirmed presence of IBV replication in all examined tissues with peak viral antigen score at 3 dpc. There were high levels of TLR3, MDA5, IFN-α, IFN-β and IL-6 gene expression were found in the challenged compared to the unchallenged control group. Findings demonstrated an early innate immune response at 1–5 days after M41 challenge. More specifically, there was a marked up-regulation of TLR3, MDA5, IFN-β and IL-6 mRNA expression in the HG, choanal cleft, turbinate and trachea.

With above findings and in an attempt to propose new quantitative parameters for assessment of protection in IBV vaccine efficacy studies, day-old broiler chicks were vaccinated with combined IBV vaccines (Mass and 793B), and at 21 days post- vaccination (dpv) chicks were challenged with IBV M41. Based on ciliary-protection test, vaccinated-unchallenged birds were had protection scores of more than 95%. Vaccinated birds were fully protected against M41 challenge (≥ 93.5 % score). The vaccinated-challenged group showed significant increases in lachrymal IgA levels at 3-5 dpc. A significantly higher viral load was found only in the trachea of vaccinated-challenged birds at 1-4 dpc. For the vaccinated groups, challenged birds had a significantly higher TLR3, MDA5, IFN-α and IL-6 expression in the turbinate, choanal cleft and trachea between 1-2 dpc. Findings from this study for the first time have provided a scientific foundation for the inclusion of quantitative early immune parameters for measurement of protection in IBV vaccination-challenge studies.

To cross-compare between IBV vaccine application methods, three strategies were applied [gel, spray, and oculonasal (ON)] in day-old broiler chicks. Birds were then challenged at 21 dpv with M41. All routes of vaccination resulted in an increase of anti-IBV IgA in the lachrymal fluid at 5 dpc compared to the control group. For gel and ON vaccination methods, for 3-21 dpv, the viral load in the turbinate, choanal cleft and trachea was higher than in the spray-vaccinated birds. Only for the oculonasal-challenge (ON-ch) group, TLR3 mRNA expression was significantly up-regulated in all tissues at 1 dpc. At 1 dpc, the mRNA expression of MDA5 and IL-6 in the all vaccinated-challenged groups showed significant up-regulation in all tissues (except for HG) compared to control group. Viral replication and the host gene signatures are likely to be related to the difference in the tissue type and method of vaccine application. It appears that the ON route of vaccination in broiler chicks provided superior protection after challenge with M41 due to higher IgA levels. This study showed that chicks vaccinated (Mass+793B) via gel were equally protected from virulent M41, and this method could be an effective commercial vaccination approach in poultry.

To date, the research onto early innate, mucosal and cellular immune responses in layer hens is limited. In this study, HALT, turbinate and tracheal tissues of adult laying hens were evaluated following heterologous vaccine application (H120 or 4/91) via drinking water (DW) or oculonasal (ON). It was found that lachrymal IgA was lower at 7 and 14 dpv compared to IgY in all groups. Viral RNA load in the HG and turbinate was significantly higher in the ON-H120 group compared to DW-H120 and both 4/91-vaccinated groups at 1–3 dpv. Host TLR3, MDA5, and IL-6 mRNA were significantly up-regulated in the HG at 3–5 dpv in the ON-H120 and ON-4/91-vaccinated groups. Expression of CD8α and CD8β indicated high up-regulation at 1–3 dpv in the turbinate and at 3–5 in the trachea. IgA mRNA expression was significantly higher in the ON-H120 and ON-4/91-vaccinated compared to DW-vaccinated groups. Generally, mucosal and cellular immune response was greater in the turbinate than the trachea for both methods of vaccination. Data demonstrated the variable role of innate, mucosal and cellular immunities in relation to vaccine type and route of vaccine administration.

Declaration

All techniques and *in vivo* experiments performed and described in this thesis were undertaken by myself in the University of Liverpool (Leahurst Campus) between April 2016 and July 2019, unless otherwise acknowledged. This work has not been submitted for any other degree or professional qualification in any other University or other institutes of learning.

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Dedication

This thesis is dedicated to my family and friends. A special feeling of gratitude to my loving parents and wife for their endless love, support and encouragement.

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List of publications and presentations

Publications in peer-reviewed scientific journals

- Mohammed Al-Rasheed, Christopher Ball, Steve Rushton, Basim Manswr, Gail Leeming and Kannan Ganapathy **(2020)**. Early host immune responses as quantitative biomarkers of protection against infectious bronchitis virus in chickens. Submitted, **Journal of Vaccine.**

Conference Publication

- Mohammed Al-Rasheed, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Innate immune responses in head associated lymphoid tissues following IBV M41 inoculation in commercial broiler chicks. XXth World Veterinary Poultry Association (WVPA) Congress, Edinburgh, UK, 4-8 September 2017. WVPA 2017-405, Poster: PO-VRD-20.

Presentations at national/ international seminar/meetings

- Mohammed Al-Rasheed, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Chicken head-associated lymphoid tissues: immune responses to virulent or attenuated infectious bronchitis viruses. University of Liverpool, Infection Biology, Departmental Seminar, 10th May 2017 (Oral presentation).
- Mohammed Al-Rasheed, Basim Manswr, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Immune kinetics in the turbinate and trachea of vaccinated and unvaccinated broiler chicks following IBV M41 challenge. Global Alliance for Research on Avian Diseases (GARAD), International Conference, Hanoi, Vietnam, 17-19 January 2018 (Oral presentation).

- Mohammed Al-Rasheed, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Innate immune responses in head associated lymphoid tissues following IBV M41 inoculation in commercial broiler chicks. University of Liverpool, Infection Biology, Faculty Poster Day, 19 June 2017 (Poster).
- Mohammed Al-Rasheed, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Innate immune responses in head associated lymphoid tissues following IBV M41 inoculation in commercial broiler chicks. XXth World Veterinary Poultry Association (WVPA) Congress, Edinburgh, UK, 4-8 September 2017 (Poster).
- Mohammed Al-Rasheed, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Innate immune responses in head associated lymphoid tissues following IBV M41 inoculation in commercial broiler chicks. University of Liverpool, Infection and Global Health Day, 16 November 2017 (Poster).
- Mohammed Al-Rasheed, Christopher Ball, Anne Forrester, Gail Leeming and Kannan Ganapathy. Immune kinetics in the turbinate and trachea of vaccinated and unvaccinated broiler chicks following IBV M41 challenge. University of Liverpool, Infection and Global Health Day, 15 October 2018 (Poster).

List of abbreviations

Ab	Antibody
ABC	Avidin biotin complex
AF	Allantoic fluid
ANOVA	Analysis of variance
Ark	Arkansas
bp	Base pairs
BSA	Bovine serum albumin
CALT	Conjunctiva-associated lymphoid tissue
CBC	Carbonate/bicarbonate coating buffer
CD_{50}	50 % Ciliostatic dose
CD 4 or 8 (+)	Cluster of differentiation 4 or 8 (positive) T cells
cDNA	Complementary DNA
CMI	Cell-mediated immune
COD	Corrected optical density
Ct	Threshold cycle
CTLs	Cytotoxic T lymphocytes
DAB	3, 3 –diaminobenzidine
DW	Drinking water
doa	Days of age
dpc	Days post challenge
dpi	Days post infection
dpv	Days post vaccination
DPX	Distyrene Plasticizer Xylene (DPX)
DNA	Deoxyribose nucleic acid
dNTP	Deoxy nucleotide triphosphate
dsRNA	Double-stranded RNA viruses
DTT	Dithiothreitol
E	Envelope protein
ECE	Embryonated chicken egg
ELISA	Enzyme linked immunosorbent assays
H&E	Haematoxylin and Eosin stain
HALT	Head-associated lymphoid tissues

HG	Harderian gland
HI	Haemagglutination inhibition
hpi	Hours post-infection
HRP	Horse-radish peroxidase
IB	Infectious bronchitis
IBDV	Infectious bursal disease virus
IBV	Infectious bronchitis virus
IFA	Immunofluorescence assay
IgA	Immunoglobulin A
IgY	Immunoglobulin Y
IgM	Immunoglobulin M
IHC	Immunohistochemistry
IL-6	Interleukin-6
IPA	Immunoperoxidase assay
Kb	Kilobase
M	Membrane protein
mAbs	Monoclonal antibodies
Mass	Massachusetts
MDA	Maternally derived antibodies
MDA5	Melanoma differentiation associated protein 5
MDV	Marek's disease virus
MEM	Minimum Essential Medium
MHC	Major histocompatibility complex
Mm	Millimetres
mRNA	Messenger RNA
N	Nucleocapsid protein
NDV	Newcastle disease virus
ng	Nanogram
NK	Natural killer cells
NLRs	NOD-like receptors
nsp	non-structural protein
°C	degrees Celsius
ON	Oculonasal
OD	Optical density

OCT	Optimal cutting temperature compound
PBS	Phosphate buffered saline
PRRs	Pattern recognition receptors
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase-PCR
REU	Relative equivalent units
RFLP	Restriction Fragment Length Polymorphism
RIG-I (RLRs)	Retinoic acid-inducible gene 1 like receptors
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT–PCR	Reverse transcription-PCR
S	Spike glycoprotein
S/P ratio	Sample/positive ratio
SAP	Shrimp alkaline phosphatase
SDW	Sterile distilled water
SEM	Standard error of the mean
SPF	Specific pathogen free
ssRNA	Single-stranded RNA
T cell	T lymphocyte (cell)
TBE	Tris borate EDTA buffer
TCoV	Turkey coronavirus
$TCID_{50}$	50 % Tissue culture infective dose
TLRs	Toll-like receptors
TMB	Tetramethylbenzidine
TOCs	Tracheal organ cultures
TPS	Tris buffered saline

Chapter 1: Literature Review

1.1 Infectious bronchitis

Infectious bronchitis (IB) is an acute and highly contagious disease that can affect broiler, breeder and layer chickens, causing economic losses for the poultry industry (Cavanagh & Gelb 2008). Schalk and Hawn first reported infectious bronchitis (IB) in the 1931. It was described as a respiratory infection prominent in chickens from two days to three weeks of age, with a high mortality rate and displayed congested respiratory pathways at post mortem (Schalk & Hawin 1931). Later, a virus was established as the causative agent of this disease and was named infectious bronchitis virus (IBV) (Beach & Schalm 1936). It was later identified in the UK in 1948 (Asplin 1948) and between 1981 to 1983 (Cook 1983, 1984). Since the first report, many serotypes and variants have been isolated and characterised (Cavanagh & Gelb 2008; Jackwood 2012; Jackwood & de Wit 2013). To date, many countries around the world have reported multiple variant IBV strains circulating in their poultry farms (Jackwood et al. 1997; Sjaak de Wit et al. 2011).

IBV has a 24 to 48-hours incubation period, and viral spread occurs rapidly by aerosol and mechanical means among chickens. The spread of IBV from an infected flock to a neighbouring flock has the potential to occur by aerosol transmission (Jackwood & de Wit 2013), also further field via migratory birds (Erbeck & McMurray 1998; Hughes et al. 2009; Promkuntod 2016). The virus is typically transmitted by aerosols created by the discharge from upper respiratory tissue. The virus also has been reported to replicate in the chicken's intestine (Ambali & Jones 1990), and other methods of transmission may include contact with faeces passed by infected chickens or faecal-contaminated fomites. Moreover, contaminated items and equipment from staff could be a significant source of spreads from one farm to another (Jackwood & de Wit 2013).

Vertical transmission has been reported (Cook 1971) but is considered of minor importance. Previous data also indicate that the virus is able to initiate venereal transmission by artificially inseminating 54-wk-old hens either with semen from IBV-infected cockerel or with IBV suspended in naïve semen (Gallardo et al. 2011).

Chickens are regarded as the most important natural hosts of IBV (Jackwood & de Wit 2013). However, several IBV-like coronaviruses have been detected in domestic and non-domestic avian species, including pheasants, peafowl, wildfowl, penguins, turkeys, wader pheasants, pigeons, quail, and Amazon parrots (Cavanagh et al. 2002; Circella et al. 2007; Dea & Tijssen 1989; Hughes et al. 2009; Wu et al. 2016). Antigenic similarity has been reported between IBV and turkey coronavirus (TCoV), which causes enteric disease in turkeys (Guy 2000). Different ages and breeds are infected by IBVs, but greater severity is evident in young chicks compared with the adults (Jackwood & de Wit 2013).

IBV initially infects the upper respiratory tract and migrates to the lower respiratory tract (Cavanagh 2007), with infection of bronchi and severe disease in young birds. Some strains of IBV cause systemic infections, and replicate in non-respiratory tissues, including the kidney (causing nephritis), oviduct (causing decreased egg production), and the gut (Ganapathy et al. 2012). In addition, the virus has been reported to cause infertility in male chickens (Boltz et al. 2007). IBV respiratory infection causes typical clinical signs such as gasping, coughing, tracheal râles, and nasal discharge (Raj & Jones 1997). Puffy, inflamed eyes and swollen sinuses may occasionally be noticed and usually persist for 5 to 7 days post-infection (dpi) , diminishing within 10 to 14 dpi in uncomplicated cases (Raj & Jones 1997). Infected chickens can also appear depressed

with a noticeable decrease in weight gain and food consumption within 3 dpi (Otsuki et al. 1990).

The severity of clinical signs is influenced by several factors associated with IBV, such as the strain type, virulence, infective dosage, sex, species and age of the chicken, immune status (vaccination) and maternally derived antibodies (MDA), secondary infections, management (such as type of housing system, vaccine programme, and biosecurity levels) and environmental stress, such as climate, dust, ammonia, and cold stress (Ganapathy 2009; Jackwood & de Wit 2013). While morbidity rates may reach 100%, the mortality rate depends on co-infection, flock age, immune status, management and environmental factors (Ganapathy 2009). Mortality rates are typically low, and possibly caused by asphyxiation induced by the blocking of the lower trachea or bronchi by mucus plugs (Raj & Jones 1997). In broilers, the nephritic form of IBV is the most common (Butcher et al. 1989; Terregino et al. 2008; Ganapathy et al. 2012) and disease stress reduces meat production by increasing the feed conversion ratio and reducing weight gain. Layers experience egg production losses or quality decline, with mild or no respiratory signs (Cook & Huggins 1986; Muneer et al. 1986), with the extent being strain-dependent (Jackwood & de Wit 2013).

Losses from production inefficiencies are usually a more significant concern than losses from mortality. Mortality peaks between 5 to 6 weeks, usually caused by a secondary bacterial infection (Chandra 1987). This association between IBV respiratory reactions and susceptibility to *Escherichia coli* (*E. coli*) or other bacteria in chickens has been studied extensively (Avellaneda et al. 1994; Cook & Huggins 1986; Hopkins & Yoder 1984; Matthijs et al. 2003; Smith et al. 1985). The damage caused by IBV to the tracheal

epithelium facilitates *E. coli* invasion and multiplication, which may result in lesions or even death, causing a major clinical and economic impact, especially in broilers (Hopkins & Yoder 1984; Smith et al. 1985).

1.2 Infectious bronchitis virus (IBV)

1.2.1 IBV structure

The causative agent of IB is infectious bronchitis virus (IBV), which is a genus *Gammacoronavirus*, family *Coronaviridae*, order *Nidovirales* (Cavanagh 2007). IBV is a positive-sense single-stranded (ss) RNA virus. It is 120 to 160 nm in diameter with typically large surface spikes of 20 nm. The shape of the virus is round to pleomorphic, with heavily glycosylated spike projections. The virus has a genome length of approximately 27.6 kilobases (kb) encoding structural proteins, spike (S1 and S2), envelope (E), membrane (M), and nucleocapsid (N), and non-structural proteins (Nsps) that are important for virus proliferation or replication.

The S protein found on the surface of the viral envelope (virion) has two cleaved forms, S1 and S2, with molecular weights of 92kDa and 84kDa, respectively. The S1 subunit contains epitopes responsible for inducing neutralising antibodies against IBV, whereas S2 is responsible for virus fusion to host cells (Belouzard et al. 2012; Ignjatovic & Sapats 2005).

The M glycoprotein is integrated into the viral lipid bi-layer with a small protruding portion on the outer surface, which is glycosylated on the N terminal domain (Rottier & Rose 1987). The E protein is a scant protein and contains highly hydrophobic transmembrane N-terminal and cytoplasmic C-terminal domains. Both the M and E

proteins are essential for virus particle formation during replication (Corse & Machamer 2003; Wilson et al. 2004).

The N protein surrounds the single-stranded positive-sense RNA genome of IBV, forming a structure termed the ribonucleoprotein (RNP) (Jackwood & de Wit 2013; Lai & Cavanagh 1997). The N protein is usually located in the cytoplasm of host cells but, as a host and viral transcription strategy for subgenomic RNA, it may also migrate to the host cell nucleus (Hiscox et al. 2001). Coronavirus N-proteins, including that of IBV, also play a role in the stimulation of cell-mediated immune responses against infection in chickens due to the presence of multiple T lymphocyte epitopes located at its carboxylic terminus (Collisson et al. 2000; Seo et al. 1997). In addition, novel linear B-cell epitope peptides, which are conserved among avian coronaviruses, have been mapped within the nucleocapsid N-terminal domain (Yu et al. 2010).

The IBV genome also has two small accessory genes, 3 and 5, which express three (3a, 3b, and 3c) and two (5a and 5b), respectively that are not essential for virus replication (Hodgson et al., 2006). Gene 3 encodes two accessory proteins, 3a and 3b, and the E protein from opining reading frame (ORF) 3c (Smith et al., 1990). The accessory proteins are involved in modulating the host response to infection (Ng & Liu 1998, 2000).

The replicase gene (gene 1) consists of ORFs 1a and 1b, located proximal to the 5' untranslated region (UTR) and the leader sequence. These encode proteins associated with RNA replication and transcription. The genes S, E, M and N are located proximally to the3' UTR and encode the proteins found in virus particles. Interspersed between

these structural protein genes are accessory genes encoding Nsps, 3b, 5a and 5b, which are not essential for replication (Armesto et al., 2012).

1.2.2 Replication of IBV

The virus replicates in the cytoplasm. IBV replication starts with the binding of S1 to receptors present in the cell membrane, namely those of α-2,3-sialic acid (Belouzard et al. 2012; Shahwan et al. 2013; Winter et al. 2006). After that, host-cell dependent proteolytic cleavage of the viral S protein during biosynthesis and the fusion of the viral envelope with the plasma membrane are observed. The virus then enters the cell either by fusion with the host plasma membrane or by receptor-mediated endocytosis (Li & Cavanagh 1992). Virus (+)ssRNA is used as a host polymerase template to synthesise viral RNA polymerase directly. The virus produces a 3' co-terminal nested set of 5 subgenomic messenger RNAs. Each mRNA has a 5' leader sequence that is joined to the mRNA during transcription. Although some of the subgenomic mRNAs are polycistronic, for the most part each mRNA encodes the protein at the extreme 5' end. The viral RNA polymerase uses the (+)ssRNA as a template for the production of a full-length negative-stranded (-)ssRNA. The negative sense genome-length and sub-genomic RNAs are used as templates for genomic and sub-genomic mRNA synthesis to synthesise other viral proteins (Zhao et al. 1993). The first step in virus assembly is the binding of N protein to viral RNA, forming the helical nucleocapsid (Weber & Schmidt 2005), alongside the integration of the M and E proteins into the membrane of the endoplasmic reticulum (ER) of the host cell (Vennema et al. 1996). Incorporation of the S protein occurs by interaction with M protein forming an S-M complex at the pre-Golgi complex (Nguyen & Hogue 1997; Opstelten et al. 1995). After budding, virus particles may undergo further morphologic changes within the Golgi, resulting in the

appearance of mature virus particles (Salanueva et al. 1999). A budding process originates viral particles

(virions) that are transported to the cell surface via the Golgi transport apparatus, and collected inside secretory vesicles, which released into extracellular space by exocytosis.

1.2.3 Strains (variant) distributions

It is well known that IBVs have poor cross-protection between serotypes, which emphasises the importance of continuous identification and surveillance (Jackwood 2012). IBV exists in the form of numerous different antigenic or genotypic types, commonly referred to as variants (Cook et al. 2012; Sjaak de Wit et al. 2011). IBV may generate new variant strains by either the process of mutation or gene recombination in the S1 gene, and may occasionally be due to the introduction of a current strain from a different region (Jones 2010). The mechanism behind the emergence of new types and variants of the virus is largely unknown (Jackwood 2012).

The first IBV strain to be isolated was the Beaudette strain (Beaudette & Hudson 1937), and later, the M41 strain was isolated in North Dakota, USA (Bracewell 1975). Despite being first identified in the USA, the classical M41 serotype and the Dutch H120 serotype, which is derived from a Dutch isolate of 1955, are the most widely used vaccine viruses (Sjaak de Wit et al. 2011). Variants have been detected around the world (Table 1.1). In the USA, the most commonly found type of IBV is Arkansas (Ark). Other commonly detected viruses in the USA are Connecticut (Conn) and Mass types (Jackwood 2012; Jackwood et al. 2010; Jackwood et al. 2005). In Latin America, in addition to the local strains (BR I and BR II), Mass type and viruses similar to the 793B

type have been reported in Brazil (Villarreal et al. 2010), several different genotypes have been isolated include Conn, Mass and Ark type in Mexico (Jackwood 2012). In the United Kingdom, a new type of IBV mostly known as 793B (also 4/91 or CR88) has been reported (Parsons et al. 1992; Adzhar *et al.* 1997 Worthington et al. 2008). In Europe, the majority of the strains are related to 793B (4/91, CR88), Italy 02, D274 (Capua et al. 1999; Worthington et al. 2008) and D1466 (Sjaak de Wit et al. 2011). The Australian 'T' strain was the first IBV strain isolated in Australia in 1962 (Cumming 1963). In China, IBV QX first identified (Yudong et al. 1998), and appears to have spread among poultry flocks in many countries (Bochkov et al. 2006; Sesti et al. 2014; Terregino et al. 2008; Worthington et al. 2008). Another IBV genotype is the Q1 which was genetically and serologically distinct from IB classical strains and reported in China from 1996 to 1998 (Yu et al. 2001). In Africa, IBV G strain was isolated in Morocco (El-Houadfi et al. 1986), and S1 sequence data shown that IBV G are very closely related to 4/91 (Jones et al. 2004). In Libya, the IBV genotype of IS/885/00-like and IS/1494/06-like (also known as Variant 2) were detected by RT PCR in broiler flocks (Awad *et al.* 2014a). In recent years, Variant 2 has spread to Poland (Legnardi *et al.* 2019) and Turkey (Yilmaz *et al.* 2016). In the Middle East, the IBV Mass, 793B, QX, Dutch strains, Variant 2, IS/885/00 and Q1 have been reported (Alsultan *et al.* 2019; Amin *et al.* 2012; Boroomand *et al.* 2011; Ganapathy *et al.* 2015).

Table 1.1. Infectious bronchitis virus types reported worldwide.

Country	Strains/Types	Reference
United State	Beaudette M41 Arkansas (Ark) Connecticut (Conn) Mass types California variant Delaware GA08 GA98	(Beaudette & Hudson 1937) (Bracewell 1975) (Jackwood 2012; Jackwood et al. 2010; Jackwood et al. 2005)
Brazil	BR I BR II Mass 793B	(Villarreal et al. 2010)
Mexico	Conn Maxx Ark	(Jackwood 2012)
United Kingdome	793B (CR88, 4/91)	(Parsons et al. 1992; Adzhar et al. 1997 Worthington et al. 2008).
Italy	Italy O2 D274 D1466 793B	(Worthington et al. 2008; Sjaak de Wit et al. 2011)
Australia	"T'' strain 793B	(Cumming 1963)
China	QX Q1	(Yudong et al. 1998) (Yu et al. 2001)
Moroccan	G strain 4/91	(El-Houadfi et al. 1986) (Jones et al. 2004)
Libya	IS/885/00 IS/1494/06 (Variant 2)	(Awad et al. 2014a)
Iran, Iraq, and Saudi Arabia (Middle East)	Variant 2 Mass 793B QX Q1	(Alsultan et al. 2019; Amin et al. 2012; Boroomand et al. 2011; Ganapathy et al. 2015)

1.2.4 Strain classification

1.2.4.1 Serotype

Based on the antigenicity of the S protein, IBV strains are classified into different serotypes, using virus neutralisation (VN) and haemagglutination inhibition (HI) testing (Toro et al. 1987). Some laboratories also use enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies (mAbs) directed against specific epitopes of the S1 protein, which can define different strains of the virus, however cross-reactions may occur between serotypes especially when sera are collected from field samples (Jackwood & de Wit 2013). The ELISA method has some disadvantages, such as poor availability of mAbs, and it is essential to produce new mAbs with each new variant (Karaca et al. 1992). Furthermore, VN and HI are not frequently used for serotyping studies due to the limited availability of the increasing number of reference sera related to the different serotypes.

1.2.4.2 Genotype

In recent years, use of DNA sequencing and genotyping based on the S1 region of the spike gene have replaced traditional serotyping methods used to demonstrate field strain identity (Cavanagh *et al.* 1999; Jackwood & de Wit 2013; Jackwood *et al.* 1992; Lee *et al.* 2000). In this type of classification, strains are typed according to the genetic characterisation of the S1 subunit, especially the S1 hypervariable region (Cavanagh 2005a). Although these classification techniques have been widely used in molecular epidemiology studies (de Wit 2000; Sjaak de Wit *et al.* 2011), many researchers have demonstrated conflicting results between serotyping and genotyping (Capua et al. 1998; Capua et al. 1999; Clewley et al. 1981; Kusters et al. 1987). A drawback of this classification method is that detection may be restricted by the primer selection, and

not all isolates from the same genotype belong to the same serotype (Cavanagh et al. 1992). Consequently, genotyping is not recommended as a standalone method, and maybe sequencing, VN and HI serotypes and *in vivo* experiments studies are all needed for IBV classification in addition to genotyping (de Wit 2000). To provide a more unified IBV genetic classification, Valastro et al. (2016) put forward a method that defines IBV strains into six genotypes comprising 32 distinct viral lineages (GI to GVI), based on the full S1 gene.

1.2.4.3 Protectotype

Protectotype or immunotype classification is the most important system from a practical point of view which are important in the efficacy of vaccine programmes in the field. Strains that induce cross-protection against each other belong to the same protectotype, such as M41 (de Wit 2000) and QX-like (Bru et al. 2017). However, vaccine strains that are not linked serologically (belong to another serotype) may still offer cross-protection, such as the live H120 vaccine that was shown to induce protection against a challenge from the Australian T strain (Darbyshire 1985). To determine the protectotype of a strain, a cross-immunisation challenge study has to be performed. However, this type of research is labour intensive, expensive, and requires a large number of birds and isolation facilities (de Wit 2000; Sjaak de Wit *et al.* 2011). Alternatively, a cross-immunisation test was conducted using tracheal organ cultures (TOCs) or oviduct organ cultures from vaccinated hens; these cultures were challenged using in vitro heterologous or homologous inoculation strains to evaluate the cross-immunity (Dhinakar Raj & Jones 1996d).

1.3 Head-associated lymphoid and respiratory tissues: anatomy, physiology, and immune responses

In poultry, the most likely lymphoid tissues that are initially exposed to vaccines or pathogenic respiratory agents, are the respiratory and lymphoid tissues in the head. Therefore, an understanding of the immune responses in the head-associated lymphoid tissue (HALT) and respiratory tissue in domestic poultry is essential, as most respiratory vaccines are delivered via spray or eye/nose drops. Infectious bronchitis virus targets the mucosal surface and paraocular lymphoid tissue (van Ginkel et al. 2008). The major inductive sites in the HALT are the Harderian gland (HG) and conjunctiva-associated lymphoid tissue, as well as lymphoid follicles distributed throughout the mucosal surfaces (Maslak & Reynolds 1995). So far, limited attention has been paid to the role of Head-associated lymphoid and respiratory tissue, including the turbinate, on immune responses to vaccine or virulent strains of IBV in chickens.

1.3.1 Harderian gland (HG)

The Harderian gland is an immune-endocrine organ located in the orbit behind the eye and is considered a major paraocular gland of domestic birds. It lies in the ventral orbit and is posteromedial to the eyeball (Kaiser & Balic 2015; Mobini 2012; Wight *et al.* 1971). The gland is relatively large in the fowl, far larger than the lachrymal gland. The HG is an ectodermally derived, exocrine tubuloacinar gland, located in the orbit behind the eye (Davison *et al.* 2008). It is loosely connected to the periorbital fascia, therefore, when the eye is removed, it may remain in the orbit (Figure 1.1) (Dyce *et al.* 2010; Olah *et al.* 2014). The gland was first defined by the Swiss physician Johann Jacob Harder in 1694. Many papers have been subsequently published regarding these glands, known as Harderian glands (Davelaar & Kouwenhoven 1976; Olcese & Wesche 1989; Scott *et*

al. 1993; Spalevic *et al.* 2012). In birds, the HG is the main orbit gland (Walls 1942) and plays a significant role in the local eye and upper respiratory immunity, in addition to its roles in lubrication and cleaning the nictitating membrane by an excretory duct (Burns 1992; Kaiser & Balic 2015).

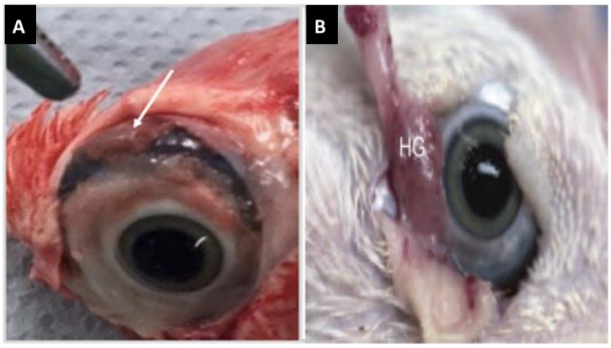

Figure 1.1. Anatomy of the Harderian gland (HG) in the chicken. (A) The dorsoventral view of the chicken's skull at necropsy with removed skin and the head's rostral aspect to the right. White arrow indicates the left side of HG. (B) By pulling the nictitating membrane with forceps (Olah *et al.* 2014), the attached HG can be withdrawn from the medial surface of the orbit.

Structurally, the gland itself is divided into head and body sections, based on differences in the surface epithelium and underlying lymphoid organisation (Olah et al. 1996). The head of the HG shows the structure of a typical secondary lymphoid organ with B cell-dependent germinal centres (GC), follicle-associated epithelium (FAE), and T cell-dependent interfollicular regions with isolated T cells, and macrophages. The body of the HG contains several B lymphocytes and plasma cells. B and T lymphocytes are grouped into separate areas depending on the level of development (Davison et al. 2008). Moreover, it probably plays a part in the local immunity of the eye and upper respiratory tract since it has a large age-dependent population of plasma cells. In addition, it is regarded as a major contributor to antibody production, and it protects

oculonasal mucosa against airborne viruses, such as IBV, ILT, and aMPV (Bang & Bang 1968; Spalevic et al. 2012; Toro et al. 1996; van Ginkel et al. 2008).

The HG is associated with mucosal immunity, studies by Bang and Bang in 1968 reported lymphocytic infiltrates in the HG of germ-free chickens, demonstrating that lymphoid tissue within the HG may be induced without microbial stimulation, although the presence of inflammatory substances in the air cannot be excluded (Bang & Bang 1968). A study by van Ginkel et al. (2009) confirmed expression of the polymeric immunoglobulin receptor after HG mRNA analysis. These findings indicate the importance of the HG in producing mucosal and systemic immunity to a pathogen such as avian influenza (AI) following the ocular-administered adenovirus (Ad5-H5) to chickens. The HG in young chicks contains single HIS-C7-positive leucocytes and small groups of positive leukocytes that are found in the connective tissue glandular lobes from 5 days of age. Moreover, B cells, macrophages, and heterophils are also present (Jeurissen et al. 1989). The number of plasma cells dramatically increases with age, and these leukocytes are found near tubular ducts and inter-alveolar connective tissue (Savage et al. 1992). Furthermore, unique to the HG is a large number of plasma cells that are capable of proliferation *in situ* (Scott *et al.* 1993).

Olah et al. (1996) documented many IgM and IgA-producing plasma cells in the HG, but rare IgY plasma cells, while Jeurissen et al. (1989) reported IgY+ plasma cells including IgY in the overlaying epithelium, but this was only observed in birds older than six weeks of age. Jalkanen et al. (1984) found more plasma cells which were cytoplasmic (c) IgY+ in 10-week-old chickens than cIgM+ and few cIgA+ cells. Lymphocytes of the

HG are of bursa of Fabricius origin and are seeded into the HG prior to hatching and do not appear to be involved in systemic immunity (Baba et al. 1988).

Albini and Wick (1974) demonstrated that 70-90% of the HG lymphocytes are from the bursa of Fabricius and 10% from the thymus. They also found that HG has a substantial number of B cells with surface immunoglobulins. Although the HG is considered the main source of IgA in lachrymal fluid, and not derived from the migration of serum IgA, IgY+ plasma cells are observed and in the lachrymal fluid contribution. IgA was detected in lachrymal fluid in healthy two weeks old chickens and levels increased with age and reached 0.2mg/ml at 15 weeks of age. IgY is initially of maternal antibody origin, decreasing over the first three weeks of life, before gradually increasing to reach approximately 2-3 mg/ml at 15 weeks of age (Baba et al. 1988).

It is still not fully understood how environmental antigens uptake, processed, and presented, and which lead to humoral antibodies in the lachrymal fluid. Survashe et al. (1979) stated that the HG's immune response is initiated in the lymphoid tissue surrounding the opening of the gland duct to the nictitating membrane. While others have submitted that, in the turkey at least, antigen uptake occurs in the lower eyelid and processing takes place in lymphoid tissue, which is connected to the conjunctiva, leading to plasma cells in the HG (Fix & Arp 1989, 1991). Using the neutralisation test, immunisation by conjunctival and intranasal routes with the H120 vaccine virus of 1-day-old chicks with high neutralisation index values resulted in immunity four weeks later, that was as significant as that achieved by vaccination of 20- and 15-day-old birds with lower maternal antibody levels. Successful vaccination coincided with significant stimulation of the HG in these age groups, increase in plasma cells and lymphocytes,

which suggests that the HG plays a significant role in the immune response to IBV (Davelaar & Kouwenhoven 1977).

1.3.2 Choanal cleft

A literature search showed that there are currently no publications on the chicken choanal cleft, with limited information available in other bird species. The shape of the avian choanal cleft is species specific. The cleft in fowl and pigeons is very long, in ducks and geese it is very short (Nickel et al. 1977). As in many avian species, there are six transverse rows of caudally directed filiform papillae on either side of the choanal cleft, and papillae were observed behind the median palate ridge. The palate of fowl and pigeons has caudally pointing papillae arranged in several transverse rows (Figure 1.2), but the goose palate has a median row of papillae and 2-3 rows of blunt papillae, these papillae were confined to the optical region (Nickel et al. 1977). There are no prior publications on immune responses to IBV in this tissue.

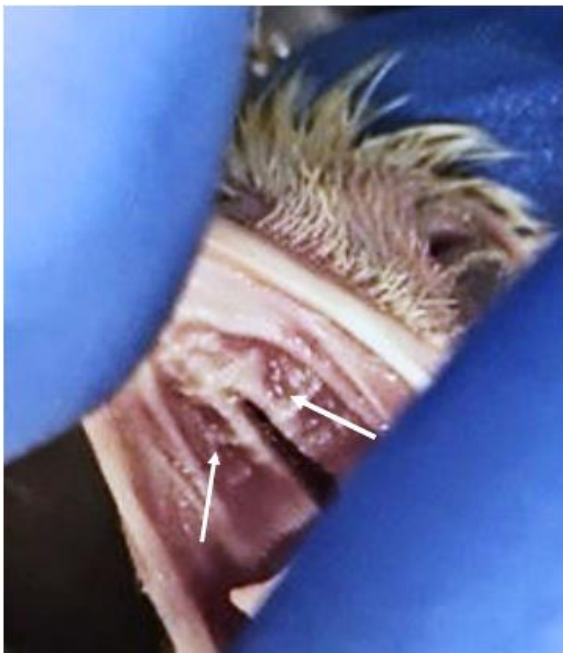

Figure 1.2. Interior of upper mandible showing choanal cleft and choanal papillae inside the mouth of a healthy chicken.

1.3.3 Turbinate

Extant reptile, mammals and birds have covered projections within the nasal cavity known as conchae or turbinate (Geist 2000). Birds and mammals typically possess an additional elaboration of the nasal cavity (Hillenius 1992; Witmer 1995). Unlike the simple conchae structure of reptiles, the avian respiratory turbinate is a highly convoluted, often scrolled structure, lined by moist mucociliated epithelium (Bang 1961, 1971). The spiral structure of the turbinate increases the superficial area of the nasal mucosa, which may prevent the entry of dust and foreign matter (Kang et al. 2013). The respiratory turbinate of birds, referred to as the anterior and middle conchae, are similar to mammalian maxilloturbinates and are situated directly in the path of the nasal epithelial mucosa and simultaneously reduce the effective distance of respiratory air from the mucosal surface (Geist 2000). Unlike the bony respiratory turbinate in mammals, avian turbinate is usually cartilaginous. The turbinates are paired in the nasal cavity, where they function as intermittent countercurrent heat exchangers (Figure 1.3) (Schmidt-Nielsen et al. 1969).

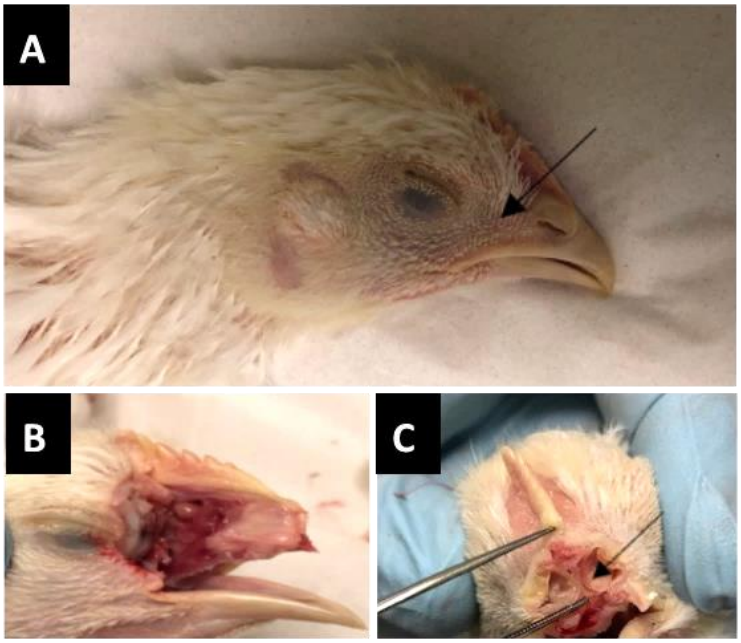

Figure 1.3. Lateral view of a chicken's head. (A) Right lateral of the head showing nasal cavity and nostril (Arrow). (B) Nasal cavity through a longitudinal sectional view of a chicken's skull. (C) Transverse view of the cross-sections from chicken's nasal cavity showing the spiral structure of a chicken's turbinate, and a pair of turbinates was located on the wall of nasal cavity which termed concha nasalis media (middle turbinate) (Arrow).

1.3.4 Trachea

Constitutive lymphoid tissue has not been described in the avian trachea. Nevertheless, infection models with *Mycoplasma gallisepticum* have demonstrated a high response by the tracheal mucosa to infection, and there is extensive lymphocyte infiltration following lymphoproliferation (Gaunson et al. 2000, 2006). Within the tracheal mucosa, CD8+ cells are found in clusters or lymphoid follicle-like structures, whereas CD4+ cells are diffuse throughout. Tracheal lesions characteristic for mycoplasma infections predominantly involve proliferating B cells (Gaunson et al. 2006). Similar responses were also demonstrated in the tracheal mucosa after infection with IBV. IBV-induced lesions are associated with large infiltration of heterophils and lymphocytes in the tracheal lamina propria. The generation of large numbers of

lymphoid follicles and the infiltration of plasma cells allows tracheal lesions to heal at approximately two weeks after infection with IBV (Kotani et al. 2000a; Kotani et al. 2000b).

1.4 Pathogenesis of IBV

IBV is primarily epitheliotropic and produces lesions after replicating in respiratory tract (nasal turbinate, trachea, HG, 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 & Jones 1997), and it can produce macroscopic and microscopic changes in the small intestine that may be associated with alteration in the S1 structure arising from mutations (Hauck et al. 2016). Although all IBV strains initially infect birds via the respiratory tract, it later disseminates to target tissues for further replication and persistence (within 18–36 hours) (Jackwood & de Wit 2013).

1.4.1 Harderian gland (HG)

Isolation of IBV from the HG has been reported following experimental infection with live attenuated H120 (Toro et al. 1996). Research to date has focused on the role of this lymphoid tissue in mucosal immunity. Chapters 3 to 6 examine the HG for IBV replication, innate and mucosal immune responses in broiler and layer chickens.

1.4.2 Turbinate

Previously, nasal turbinate organ culture inoculated with six different IBV strains (H52, H120, M41, Connecticut, Australian T strain and British field strain HV-10), resulted in maximum viral titres between 48-72 hours post infection (Darbyshire et al. 1978). The antigen was detected in the turbinate organ culture after vaccination with live IBV H120 (Darbyshire *et al.* 1976). Dolz et al. (2012) also revealed the presence of viral RNA of

IBV Italy 02 serotype in the nasal turbinate prior to detection in the trachea using in-situ hybridisation. The turbinate is likely to be the first defensive barrier of the respiratory tract when infection comes through air. To enhance our knowledge on the role of turbinate in unvaccinated and vaccinated birds, a number of experiments were carried out in broiler (Chapter 3 to 5) and layer (Chapter 6) chickens.

1.4.3 Trachea

It has previously been observed that IBV replication occurs in ciliated and mucus-secreting epithelial cells of the trachea (Benyeda et al. 2010; Nakamura et al. 1991; Owen et al. 1991; Yagyu & Ohta 1990) and IBV is frequently isolated from trachea (Janse et al. 1994; Lee et al. 2002; Otsuki et al. 1990). The persistence of the virus in the trachea is strain-dependent. For example, infection by strain G in the upper respiratory tract demonstrates the highest viral titres at 3 dpi, and viral isolation was observed up to 14 dpi (Ambali & Jones 1990), whereas 793B was isolated from the trachea of infected SPF chicks up to 7 dpi (Raj & Jones 1996a). Tracheal damage in terms of ciliary activity has been suggested to be dependent on IBV strain virulence (Dhinakar Raj & Jones 1997; Otsuki et al. 1990). Based on the cilia-stopping test, the QX-like and 793B strains proved to be the least virulent, while the effect of M41 infection was more severe (Benyeda et al. 2009). In Chapters 3 to 6, the role of the trachea in comparison to HALT and respiratory tissues was studied.

1.4.4 Oviduct

IBV may replicate in the oviduct epithelium in young chicks (Crinion & Hofstad 1972a) and in laying hens (Jones & Jordan 1971). IBV infection in young pullets can lead to permanent damage to the oviduct, resulting in a significant decrease in both egg number and quality, and the production of “false” layers who are unable to lay eggs

due to the formation of cysts within the oviducts. False layers are the result of infection of 2-week-old females due to the resulting damage to the oviduct (Boltz *et al.* 2004; de Wit *et al.* 2011b; Ganapathy 2009; Jones & Ambali 1987; Worthington *et al.* 2008). IBV has been detected in testes, suggesting a potential tissue tropism for some IBV strains and the resulting risk of infertility in males (Boltz et al. 2004; Gallardo et al. 2011; Villarreal et al. 2007). The pathology of IBV infection in the male reproductive tract has not been well reported.

1.4.5 Kidney

In addition to replicating in the upper respiratory tract, nephropathogenic strains of IBV may also primarily replicate in in epithelial cells in the kidney from the lower nephron to the collecting duct (Chen & Itakura 1996). It is likely that high titres of the virus in the kidney do not correlate well with overt kidney disease. For instance, infection with Moroccan G strain resulted in similar titres in both the kidney and trachea, although no gross changes were noted in the kidney (Ambali & Jones 1990). Nephropathogenic strains produce fewer respiratory signs and lesions (Ganapathy et al. 2012; Glahn et al. 1989; Terregino et al. 2008; Ziegler et al. 2002) but can induce high mortality in younger birds (Cook et al. 2001; Liu et al. 2006). However, other strains such as Moroccan Italy 02 (coded, IBV/MN, IBV/TU and IBV/RA) cause slight or no renal lesions or mortality in one-day-old SPF chicks under experimental condition (Khataby et al. 2016).

1.4.6 Other tissues

IBV replicates in epithelia beyond the respiratory tract (Raj & Jones 1997), and has been isolated from other tissues, including lung, air sacs, oesophagus, proventriculus, duodenum, jejunum, liver, spleen, bursa of Fabricius, caecal tonsils, ileum, rectum,

cloaca, and semen (Ambali & Jones 1990; Cook 1971; Dolz *et al.* 2012; Ganapathy *et al.* 2012; Lucio & Fabricant 1990; Otsuki *et al.* 1990; Raj & Jones 1996a).

1.5 Immune responses

Lymphoid tissues are either of epithelial (e.g., thymus and the bursa of Fabricius) or mesenchymal (e.g., spleen and bone marrow) origin and are occupied by haematopoietic cells via the blood. The primary lymphoid organs (thymus and bursa), are colonised by stem cells of haematopoietic origin that mature in situ to become immunologically competent T (thymus) and B (bursa) cells. This process involves developing the T and B cells to ignore self-antigens but to respond to foreign antigens. These immunologically mature cells then re-enter circulation and colonise the peripheral lymphoid organs such as HG and nasal-associated lymphoid tissues (Figure 1.3). The chicken immunity can be divided into the following, i) passive, ii) innate, iii) humoral, iv) mucosal (local) and v) cellular.

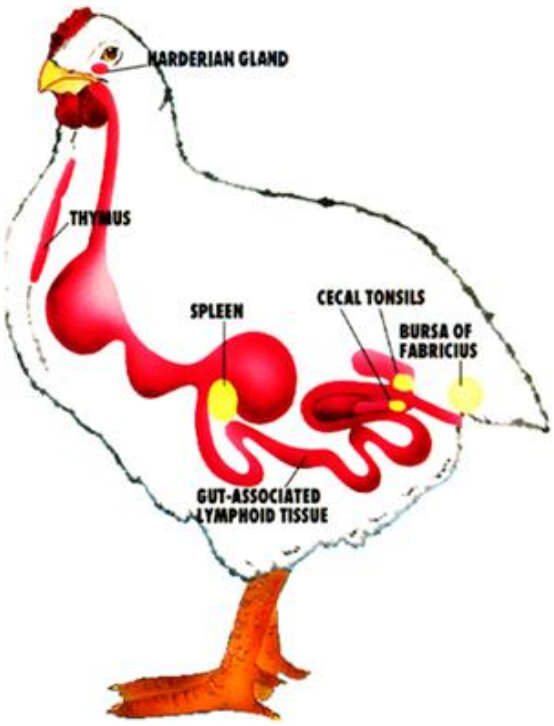

Figure 1.3. Diagram of avian immune organs (Shaker 2014).

1.5.1 Maternal derived antibodies (MDA) (passive immunity)

High levels of MDA may delay and decrease the serological response to vaccination or infection at the time of vaccination or infection used on the day of the hatch (de Wit 2000; Pensaert & Lambrechts 1994), therefore establishing when MDA deceases is essential. Broadfoot and colleagues demonstrated that passive immunity could protect young chicks (1 to 18 days of age) from abnormal oviduct development when challenged with virulent field strains of IBV (Broadfoot et al. 1956). MDA can provide protection against IBV infection in newly hatched chicks at 1 to 7 days of age (Mockett et al. 1987). In another study, chicks hatched with high MDA were shown to have significant protection against IBV challenge (IBV-Mass strain) at 1 day old but not at 7 days old. MDA protection was significantly associated with high concentration of local antibody in the respiratory tract but not with high serum antibody levels (Mondal & Naqi 2001). Vaccination of 1-day-old chicks did not result in detectable antibodies by virus neutralisation, whereas vaccination of 6 to 20-day-old hens with lower levels of maternal derived IBV-antibody resulted high detectable antibodies (Davelaar & Kouwenhoven 1977). Live vaccination of day-old chicks resulted in a rapid decrease in MDA, due to the binding and partial neutralisation of the vaccine virus (Mondal & Naqi 2001). Moreover, maternally derived IBV (D388 serotype, QX genotype) neutralising antibodies provide partial protection against tracheal damage and high protection against viral replication in the kidney (de Wit *et al.* 2011b).

1.5.2 Innate immunity

Innate immunity comprises several factors that resist invasion by external agents; these include physical barriers such as the skin and mucous membranes, soluble factors like lysozyme, complement and acute-phase proteins, and cells such as granulocytes,

macrophages, dendritic cells and natural killer (NK) cells. The main features of innate immunity are a lack of specificity and immunological memory (Raj & Jones 1997). In many respiratory diseases, such as infectious bronchitis, heterophils constitute the first line of defence against infectious agents and are the first cells to be recruited to the site of infection, along with other lymphocytes in the HG and trachea of IBV-infected tissues (Nakamura et al. 1991; Raj & Jones 1997; Toro et al. 1996). Macrophages are a chicken's main defence against infection and are distributed in the infected tissue. Following initiation of an inflammatory response, the number of heterophils and macrophages dramatically increase, from 24 to 72 and from 24 to 96 hours post infection (hpi), respectively (Fulton et al. 1993). Along with dendritic cells, macrophages produce cytokines and chemokines, which trigger the local inflammatory phases (Hawkes et al. 1983). These initial-stage inflammatory reactions to IBV infection are activated by the rapid influx of proinflammatory cytokines, involved in activating innate immune responses by recruiting polymorphonuclear cells and monocytes to the main site of viral infection. The overexpression of these cytokines might be connected with tissue damage (Kameka *et al.* 2014).

The downstream immunomodulatory cascade is activated by Toll-like receptors (TLRs). These quickly recognise conserved structures in infectious agents through pattern recognition receptors (PRRs). The PRRs are present on the cytoplasmic surfaces of immune cells, such as macrophages, dendritic cells, lymphocytes, and several non-immunological cells, such as endothelial cells, mucosal cells, and fibroblasts. In chickens, there are four essential PRRs: TLRs, retinoic acid-inducible gene (RIG-I), regulatory inducible gene-like receptors (RLRs), and NOD-like receptors (NLRs) (Chhabra et al. 2015a) (Figure 1.4).

In-depth research on PRRs and the immunological parameters that accompany their expression showed that TLR3 induces the production of interferon-I (IFN-I; α and β) through viral RNA double-stranded sensing (Kint *et al.* 2015). These IFNs then actively induce adjacent cells to express high levels of antiviral proteins, stimulating NK cells and macrophages to generate an antiviral response. Up-regulation of pro-inflammatory cytokines such as IL-6 and IL-1β are produced during IBV infection. This coincides with the highest viral RNA load and greatest severity of microscopic lesions, indicating a key role of these cytokines within high viral load and the lesion development in the trachea and kidney tissues (Chhabra *et al.* 2015a; Okino *et al.* 2014) (Figure 1.4).

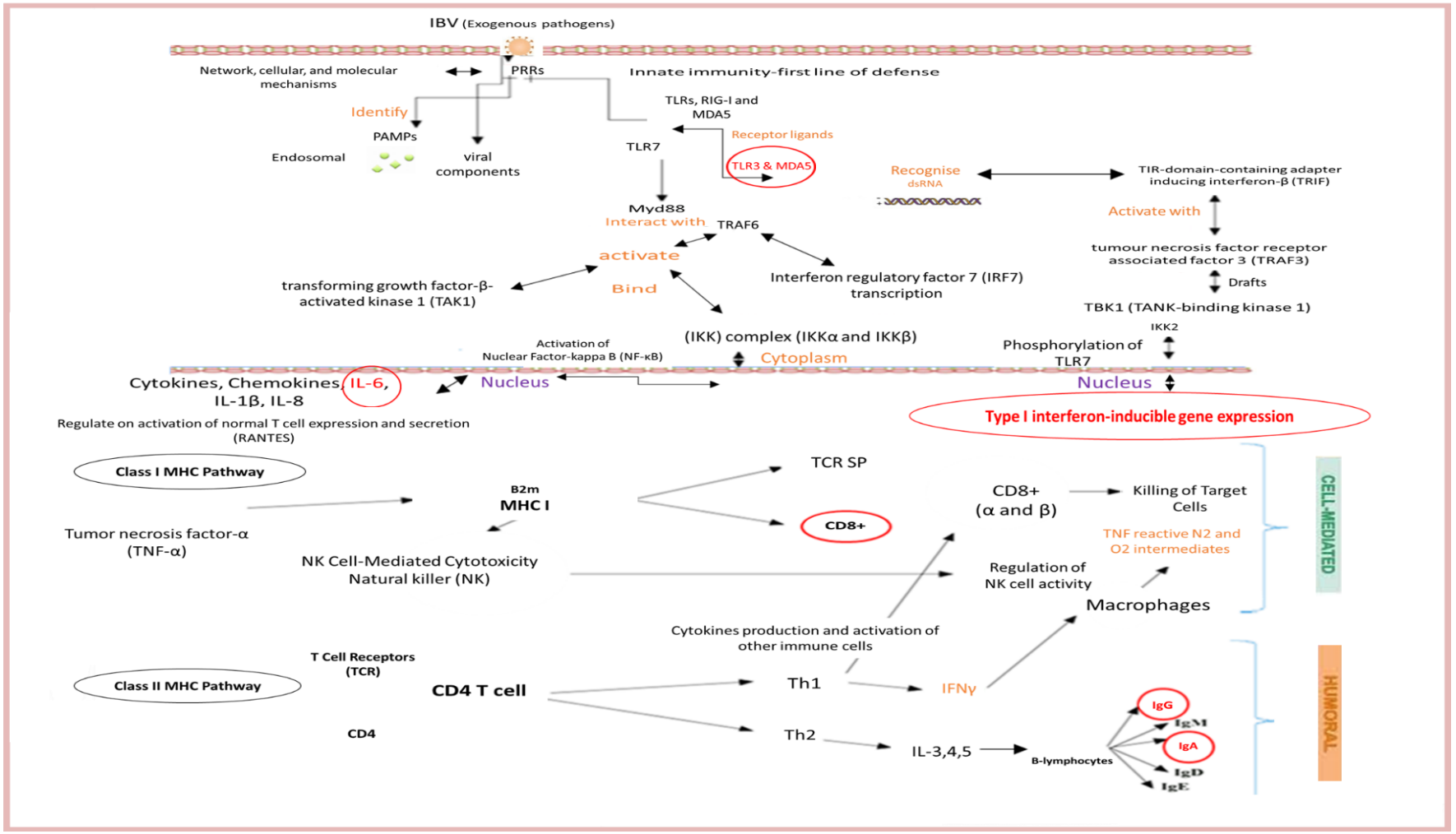

Figure 1.4. A proposed model of the cellular and molecular kinetics of innate immunity following infection or immunisation of IBV in chickens. IBV antigen is primarily recognised by two independent innate mechanism including dsRNA derived from viruses or virus-infected cells (TLR3), short 5'ppp dsRNA (RIG-I) and long dsRNA (MDA5). The red colour showed that target genes or cells are examined in this study.

1.5.3 Acquired immunity

Unlike the innate immune response, acquired (adaptive) immunity results in the activation of antigen-specific effector mechanisms. Such mechanisms include B-cells (humoral response), T-cells (cellular response), macrophages, and the production of memory cells, which play an important role in antiviral immunity against respiratory diseases (Raj & Jones 1997). Dendritic cells play an important role in the initiation of the adaptive immune response, as they are the only cells that can activate naïve T-cell subsets by their high levels of MHC molecules and co-stimulatory activity (e.g., expressing CD80/86) (R. Juul-Madsen et al. 2014). Both T and B cells, may be stimulated by the antigen-presenting cells (APCs) to help counter infection and support recovery (Chhabra et al. 2015a). Antigen-presenting cells, including macrophages and dendritic cells, 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 1.4).

1.5.3.1 Humoral immunity

The role of the bursa of Fabricius helps to differentiate the humoral immune response from the cell-mediated immune reaction (Davison *et al.* 2008; Schat *et al.* 2014). Upon immunostimulation, B cells differentiate into plasma cells and secrete antibodies either in the presence or the absence of T-helper cells. The humoral immune response to IBV has been extensively studied since the first detection of the virus (Cavanagh 2007; Chhabra *et al.* 2015b; Dhinakar Raj & Jones 1996d; Ignjatovic & Galli 1994; Okino *et al.* 2013). Although humoral immunity to IBV is essential in the systemic immune system, several studies have shown that circulating antibody titres do not correlate with protection against IBV (Darbyshire & Peters 1985; Raggi & Lee 1965). Humoral

antibodies to IBV infection can be continually measured in serum by serological testing with ELISA, HI, or VN (Dhinakar Raj & Jones 1996d). It has been previously reported that the differences in the humoral antibody response following vaccination are related to vaccination dose and application route (Okino et al. 2013; Toro et al. 1997).

The first serum antibody detected after IBV infection is IgM (Gillette 1974). Its concentration is maintained in the blood for a shorter period than other antibody classes following administration of a virulent (Mockett & Cook 1986) or an attenuated IBV (Martins et al. 1991). Significant amounts of IgM were found between 5 and 18 dpi in serum, with the highest titres at 8 to 10 dpi after IBV M41 infection (Mockett & Cook 1986). Thus, IgM could be used as a tool for early detection of IBV infection (Mockett & Cook 1986).

IgY in chickens is the major circulating immunoglobulin, and the response kinetics are very different from the IgM response. Anti-IBV IgY can be detected in serum as early as 3 dpi, peaking at about 21 dpi and after this time titres decline towards the 63 dpi (Mockett & Darbyshire 1981). After reinfection, serum IgY levels were much higher than those observed in the primary infection response (Mockett & Darbyshire 1981). Thus, the primary IgY persisted for a longer period than IgM following IBV infection (Mockett & Cook 1986).

1.5.3.2 Mucosal (local) immunity

The role of local antibodies in the defence of mucosal surfaces against IBV diseases has been well documented (Dhinakar Raj & Jones 1996a, 1996b; Gomez & Raggi 1974; Nakamura *et al.* 1991). All the local immunoglobulins (IgM, IgA, and IgY) in the lachrymal fluids are produced by the HG, which provides local protection in the upper

respiratory tract via these immunoglobulins (Baba *et al.* 1988). It has also been recently reported that simultaneous application of Ma5 and 4/91 vaccine strains induces high levels of immunoglobulins (IgA and IgY) in the upper respiratory tract (URT) washings and high levels of CD8+ T cells in the HG (Smialek *et al.* 2016).

Following IBV infection of the tracheal epithelium, IgM, IgA, and IgY in the trachea have been demonstrated (Nakamura et al. 1991). In the trachea of IBV infected chickens, IBV-specific IgA antibodies have been detected in the lamina propria, tracheal washes, and epithelial cells (Joiner et al. 2007). IBV-specific IgA antibodies have also been reported in lachrymal fluid, which correlated with resistance to IBV reinfection (Davelaar et al. 1982; Gelb et al. 1998). IBV-specific IgA in the lachrymal fluid was initially detected 10 days post vaccination (dpv) with attenuated Ark DPI-type IBV live vaccine. However, no further significant increase in IgA was detected after challenge of the vaccinated chickens with Ark-IBV isolate AL/4614/98, clarifying the likely role of neutralising antibodies in the lachrymal fluid at the time of challenge, decreasing the efficiency of IBV infection and thus subsequent stimulation of the IgA antibody response upon challenge (Joiner et al. 2007).

Following infection with virulent strains, IBV-specific IgA and IgY have been detected by the use of class-specific monoclonal antibodies in enzyme-linked immunosorbent assays (ELISA) in lachrymal fluid, tracheal washes, oviduct washes, duodenal and caecal contents (Dhinakar Raj & Jones 1996b; Ganapathy *et al.* 2005; Hawkes *et al.* 1983; Meir *et al.* 2012), and in the HG using enzyme-linked immunospot (ELISpot) (van Ginkel et al. 2008). Ocular vaccination with a live-attenuated H120 vaccine induced IgA-positive plasma cells in the HG at 14 dpv (Davelaar & Kouwenhoven 1981; Davelaar et al. 1982),

whereas a pathogenic Arkansas strain induced them earlier at 9 dpi. Dhinakar Raj and Jones (1996a) reported that lachrymal fluid showed the highest IgA and IgY antibody concentration on 7 dpi, but while IgA decreased to an insignificant level by 17 dpi, IgY was still measurable at 23 dpi. IgA and IgY levels in lachrymal fluid appear to correlate with protection against IBV or may be indicative of effective IBV vaccination (Okino et al. 2013). Levels of expression of the different immunoglobulins (IgM, IgY, and IgA) have been reported to be dependent on the particular chicken line (Nakamura et al. 1991). The IBV-specific IgY responses are less protective than IBV-specific IgA antibodies found in lachrymal fluids (Toro & Fernandez 1994). Furthermore, IBV-specific IgA antibodies have also been identified in lachrymal fluid and later serum, suggesting that IgA is essential to neutralise IBV on mucosal surfaces and is believed to have a local function in IBV control (Davelaar et al. 1982).

Collectively, humoral and local immune systems play an essential role in the control of IBV infection. The HG considered the main eye-associated lymphoid tissue, and the conjunctiva of the lower eyelid, are involved in the local immunity in the eye. As the HG secretions (secretory immunoglobulins) protect the upper respiratory tract (Gurjar et al. 2013), removal of the HG resulted in a decreased level of protection against IBV infection in day-old vaccinated chicks (Davelaar & Kouwenhoven 1980).

Humoral antibody levels do not closely correlate with tissue protection, but local antibodies may contribute to the protection of the respiratory tract (Ignjatovic & McWaters 1991; Raggi & Lee 1965). Some studies have shown that local antibodies play a role in preventing re-infections from respiratory pathogens (Hawkes *et al.* 1983; Holmes 1973) and that the HG contributes to this local protective immunity (Hawkes

et al. 1983; Toro & Fernandez 1994; van Ginkel *et al.* 2008). However, the mechanism of local antibodies in preventing the re-infection is not clear. Gelb et al. (1998) found that some chickens with high lachrymal fluid IBV antibody titres were IBV susceptible and some low lachrymal fluid antibody titres chickens were protected, suggesting that mechanisms other than lachrymal fluid-mediated immunity in viral clearance following infection are important.

1.5.3.3 Cellular immunity

The cellular immune system is controlled by the thymus. The thymus gland in chickens is composed of multiple lobes located along the side of the neck and extending into the thoracic cavity. The thymus is crucial for the maturation of T lymphocytes, the principal cells of cellular immunity. Other cells relevant to the cellular immune response include macrophages, dendritic cells, NK cells, and effector cells of antibody-dependent cellular cytotoxicity (Sharma 1997).

The cell-mediated immune (CMI) response is critical in the recovery from IBV infection (Collisson et al. 2000). After infection with virulent IBV 793B, CD4+ and CD8+ cells were shown in sections of the trachea, lung, and kidney (Dhinakar Raj & Jones 1996a). The CD4+ and CD8+ response was detected early at 3 dpi, and began decreasing at the same time point as the viral load declined (Seo et al. 1997), which suggests that the CMI response could play an important role in the early phase of limiting an IBV infection (Seo & Collisson 1997). CD8+ cells are essential for controlling many virus infections (Jamieson et al. 1987; Reddehase et al. 1988; Whiteside et al. 1993). It has been demonstrated that adoptive transfer of IBV specific CD8+ memory T cells collected at 3 to 6 weeks post-infection can protect naïve chicks from acute IBV infection (Pei et al. 2003). Conversely, adoptively transferred CD4+ cells do not appear to be essential in

the early recovery of IBV infection in chickens (Seo et al. 2000), but are important for long-term virus control (Reddehase et al. 1988; Thomsen et al. 1996). For vaccinated birds, Okino et al. (2013) reported the expression of various cytokine genes in the trachea following inoculation with different doses of live H120 vaccine. Their results found that protection against virulent M41 challenge was determined by CMI response levels. Seven days after infection with IBV-M41, in the trachea there was induction of CD8α mRNA which was associated with the greatest viral load and microscopic lesion scores (Okino et al. 2014). The role of memory T cells in the respiratory epithelial tissues, such as turbinate and trachea, needs further examination, particularly in the turbinate where there is currently a lack of information. Part of the Chapter 6 investigated the role of T cells in the respiratory epithelial tissues, such as turbinate and trachea.

1.6 Diagnosis of IB

1.6.1 Clinical diagnosis

1.6.1.1 Clinical signs

Chickens of all ages and breed types are susceptible to IBV infection, but the magnitude and severity of infection is more pronounced in young chicks than adults (Bande et al. 2016). Typical respiratory clinical signs include tracheal râles, coughing, sneezing, gasping, difficulty breathing and nasal discharge (Raj & Jones 1997). In uncomplicated cases, these signs can last for 5–7 days and disappear within 10–14 days (Raj & Jones 1997). Other signs include anorexia, huddling under heat sources, discoloured diarrhoea, watery eyes, swollen sinuses, and weight loss (Jackwood & de Wit 2013). Signs can depend on factors such as strain virulence, age of the bird (Ganapathy 2009), breed, type of housing system, vaccine programme, and co-infections (Jackwood & de

Wit 2013). IBV has a relatively short incubation period in flocks, with reports of 18–48 hours and clinical signs may last for about 2-3 weeks (Ignjatovic & Sapats 2000). After 3–5 dpi, the virus titre peaks, then rapidly decreases in uncomplicated infections. The virus grows primarily in the upper respiratory tract, subsequently spreading to other, non-respiratory tissues (Jones & Ambali 1987; Lucio & Fabricant 1990). Secondary infections from other bacterial and viral pathogens, such as *E. coli*, can prolong respiratory signs (Nakamura et al. 1992). In some cases, co-infection with *Mycoplasma synoviae* can result in egg abnormalities (Feberwee et al. 2009). If chronic respiratory disease (CRD) develops, it may last for several weeks, with mortality ranging between 5% and 25% (Fabricant & Levine 1962).

Cold climate, high-protein diet and chicken species are factors that can increase mortality rates following infection with nephropathogenic strains (Butcher et al. 1989). Wet litter can be present, which is usually due to increased water intake following infection (Irvine et al. 2010). Occasionally, mortality occurs within 4-5 days after infection, with the mortality risk subsiding by day 12 (Cumming 1963).

IBV infected layer birds may have lower egg production and quality, or a loss of shell pigment (Butcher et al. 2011; Cavanagh & Naqi 2003). Further to the clinical respiratory signs, reduction in egg production can be up to 70% (Box et al. 1980; Box & Ellis 1985). Some eggs can also have soft, misshapen, or rough shells, watery albumin and decreased hatchability (Cumming 1969; Ganapathy 2009). Production levels can also be influenced by factors such as the IBV strain serotype and host immunity (van Eck 1983). False layers may result from infection of 2-week-old hens, due to the resulting damage to the oviduct (de Wit *et al.* 2011b; Ganapathy 2009; Worthington *et al.* 2008).

1.6.1.2 Gross pathology lesions

Following vaccination or infection, the HG after is described as enlarged and inflamed at post mortem examination (Davelaar & Kouwenhoven 1976).

Congestion, the presence of catarrhal exudate, and mucosal hyperaemia in nasal turbinate and trachea were observed in chicks and 40-week-old SPF hens after infection with IBV Italy 02 (Dolz et al. 2012). The incidence of nasal exudate in infected chickens has been used to assess the severity of disease in different lines of chickens (Parsons *et al.* 1992; Raj & Jones 1996a). In severe cases, the virus spreads from the trachea and causes significant exudation and necrosis in the air sacs (Cavanagh & Naqi 2003). Air sacs may become foamy, cloudy, and sometimes contain yellow caseous exudate during acute infection (Jackwood & de Wit 2013; Ziegler et al. 2002). Caseous casts can be seen blocking the lower trachea or bronchi, which may result in the death of the bird by asphyxiation. Pulmonary congestion and small areas of pneumonia may be observed in the lungs (Raj & Jones 1997).

In the oviduct gross changes can vary, from an underdeveloped structure to a blind sac projecting forward from cloacae (Jones & Jordan 1972). In addition, many publications have reported that IBV infection in young female chickens can lead to cystic oviducts (Awad *et al.* 2016a; Benyeda *et al.* 2009; Chew *et al.* 1997; Crinion & Hofstad 1972b; de Wit *et al.* 2011b), or a decrease in the weight and length of oviducts, potentially accompanied by a decreased ovary size (Sevoian & Levine 1957). It has also been reported that excessive amounts of exudate in the oviduct can occur (Awad et al. 2016a; Ganapathy 2009). The severity of pathological lesions in the oviduct may vary, based on IBV strain and age of the hen (Cavanagh & Naqi 2003).

Kidneys infected with nephropathogenic IBV strains can become swollen and pale, and the tubules and ureters can be distended with urates, causing increased water intake and diarrhoea (Cumming 1963; Ganapathy 2009).

Infection with the IBV 793B strain was initially associated with deep pectoral myopathy in chickens, and occasionally prominent fascial haemorrhage in both deep and superficial pectoral muscles (Gough *et al.* 1992; Raj & Jones 1996a). Infection with IBV, such as QX-like IBV or Q1, may cause proventriculitis in broiler chickens, SPF chicks or in young layer flocks (Ganapathy et al. 2012; Yu et al. 2001).

1.6.1.3 Histopathology

1.6.1.3.1 Harderian gland (HG)

Following conjunctival and intranasal inoculation with IB vaccine virus at both one and 20-days-old, a significant increase in the number of plasma cells, and an increase in vascularisation have been observed in the stroma of HG lymphoid tissue of chickens. This was followed by marked formation of lymphoid follicles in chickens at 7-21 dpv (Davelaar & Kouwenhoven 1976). In addition, degeneration of plasma cells was observed in the HG of broilers at two weeks post vaccination with H120 or H52 vaccine virus (Davelaar & Kouwenhoven 1977). Epithelial vacuolation and a 25–30-fold increase in the number of Russell body (RB) cells seen in the HG. The increase was a result of ocular infection with H120 IBV (Survashe et al. 1979). Toro and colleagues also reported partial damage to the HG following infection with H120 IBV serotype as demonstrated by the appearance of RB cells and by tubular epithelial cell exfoliation that occurs concurrently with the presence of detectable IBV (Toro et al. 1996).

1.6.1.3.2 Turbinate

Characteristic lesions of the degenerative and hyperplastic stages were observed in nasal turbinate at 3 to 7 dpi following exposure to Italy 02 IBV serotype. The lateral nasal gland showed mild lymphocytic interstitial infiltration and necrosis of glandular epithelial cells (Dolz et al. 2012). Respiratory tissues, particularly the nasal turbinate, needs further investigation.

1.6.1.3.3 Trachea

Lesions in the trachea are the most common and in infected chicks consist of oedema with de-ciliation, rounding, or sloughing of the epithelial cells following infection (Nakamura et al. 1991). The severity and persistence of lesions might differ between different IBV strains (Benyeda et al. 2010). Infiltration of heterophils and lymphocytes can be seen within 18 hours following infection, and regeneration of the epithelium starts within 48 hours. The lamina propria may be infiltrated by lymphoid cells and a formation of large number of germinal centres, which can be seen from 7 days post infection (Cavanagh & Gelb 2008).

1.6.1.3.4 Oviduct

Oviducts of adult IBV-infected hens demonstrated a decrease in height and loss of epithelial cells, in addition to glandular dilation and infiltration by lymphocytes, plasma cells and heterophils, especially around the blood vessels. Fibroblasts and plasma cells in the lamina propria with oedema of the submucosa of the oviduct have also been observed (Crinion et al. 1971; Ignjatovic & Sapats 2000). Following IBV M41 infection, oviduct cysts with a thin microscopic cyst wall, but an intact ciliated epithelium internal layer was observed (Crinion et al. 1971). The IBV M41 serotype produced the greatest number of changes, followed by the Australian T strain (Crinion & Hofstad 1972a).

However, M41 varied in its ability to produce microscopic changes in the oviduct. SPF or immunocompromised chicks can become infected, and oviduct damage is more severe and permanent in these birds (Crinion et al. 1971).

1.6.1.3.5 Kidney

The kidney is the second most commonly investigated tissue when IB is suspected. Typical histological changes in the kidney present as interstitial nephritis (Riddell 1987). The virus causes granular degeneration, vacuolation, and desquamation of the tubular epithelium, as well as excessive infiltration by heterophils in the acute stage of disease. Necrotic foci may be seen during the regeneration of the tubular epithelium, and the inflammatory cell population changes to lymphocytes and plasma cells during recovery. In urolithiasis, the ureters associated with an atrophied kidney are distributed and often contain large calculi in the ureters (Riddle 1987; Siller & Cumming 1974).

Characterisation of lesions in head-associated lymphoid (HG and choanal cleft) and respiratory tissues (turbinate and trachea) is important for an increased understanding of IBV pathogenicity.

1.6.2 Virus isolation (VI)

IBV can be isolated from infected birds using TOCs (Cook 1984; Cook *et al.* 1976), SPF embryonated chicken eggs (ECE) (Darbyshire 1978; Ganapathy 2009), or primary chicken kidney cell culture (Otsuki et al. 1979). IBV can be isolated from OP, CL or tracheal swabs, and tissue samples including the trachea, lungs, oviduct and kidneys (Jackwood & de Wit 2013). However, these isolations are not sufficient to confirm the presence of IBV and further confirmation is required by other methods. Therefore, isolation performed in combination with other tests is used to maintain the sensitivity

(de Wit 2000). Isolation can be hampered by a mixed infection with different strains of IBV, or another virus (de Wit 2000). Isolation of IBV takes a long time and is more expensive than RT-PCR. Hence, alternative techniques, such as IFA, immunohistochemistry assay (IHC), antigenic ELISA, and RT-PCR, could be successful for antigen or nucleic acid detection, rather than detection of infectious particles.

1.6.3 Virus antigen detection

1.6.3.1 Immunohistochemistry assay (IHC)

Immunoperoxidase (IPA) and immunofluorescence (IFA) assays are two significant histochemical staining techniques for infected tissue and/or cells to identify and confirm the presence of IBV antigen (Bande et al. 2016). Both methods are based on antigen-antibody reactions (Bezuidenhout et al. 2011; S. Arshad & Al-Salihi 2002). A specific anti-IBV- peroxidase-labelled antibody is used to detect the antigen; dyes activated by the peroxidase allow visualisation of the signal. The avidin-biotin complex (ABC), has been successfully used on tissue samples to detect IBV antigen (Abdel-Moneim et al. 2009). Light microscopy can be used to evaluate the slides, and can be stored for longer periods compared to IFA, where staining can fade if samples are not kept frozen (de Wit 2000). However, IPA requires a few days per reaction, and is sensitive to non-specific background staining by endogenous peroxidase naturally present in the sample. Removing endogenous peroxidase in tissues is preferable during the procedure to avoid non-specific staining (de Wit 2000).

1.6.3.2 Detection of IBV genome

In view of their increased sensitivity, molecular methods such as RT-PCR, real-time PCR and Restriction Fragment Length Polymorphism (RFLP) have replaced conventional virus detection methods in IBV diagnosis (Adzhar *et al.* 1997; Adzhar *et al.* 1996).

RT-PCR exhibits moderate sensitivity when performed on samples directly obtained from infected chickens (Jackwood et al. 1997). Sensitivity may be improved by performing one or two passages in embryonic eggs or TOCs to boost viral load followed by RT-PCR identification of the IBV genome (de Wit 2000; Jackwood & de Wit 2013). Primer pairs are typically designed for amplifying highly conserved segments of the M and N viral protein genes (Andreasen et al. 1991; Falcone et al. 1997). Universal oligonucleotide primers corresponding to the S1 gene are designed to work with many IBV types with exception of D1466 strain (Adzhar et al. 1996; Worthington et al. 2008). Nested RT-PCR is more sensitive than the conventional RT-PCR because of the addition of a second amplification step using DNA amplified from the first PCR (Cavanagh *et al.* 1999). Concurrently, qRT-PCR has also been used in the detection of IBV genome (Marandino *et al.* 2016; Roh *et al.* 2014). There are several qPCR assays using different techniques, as SYBR Green I real-time RT-PCR (Fellahi et al. 2016) and Taq Man probe methods (Callison et al. 2006). Due to risk of contamination during the test and high sensitivity, the possibility of false-positive results is present in nested RT-PCR (de Wit 2000; Jackwood & de Wit 2013). After detecting the IBV genome, PCR sequencing techniques, such as restriction fragment length polymorphism (RFLP) or hybridisation with IBV specific probes can be applied (Binns et al. 1985; Jackwood et al. 1992; Moore et al. 1998). However, a positive RT-PCR result does not distinguish between infectious and non-infectious virus particles and is not sufficient for diagnosis. Sequences of the RT-PCR product can be compared with other relevant sequences for differentiation of the virus (genotyping) (Jackwood & de Wit 2013).

1.6.4 Detection of IBV- specific antibody (serological)

1.6.4.1 Haemagglutination inhibition test (HI)

The HI test identifies antibodies to the IBV S1 spike glycoprotein, and the test requires 7-14 days from when the samples are taken to confirm the detection of antibody (de Wit *et al.* 1997). The HI test is strain and serotype-specific (de Wit 2000; de Wit *et al.* 1997). It can limit the use of HI to monitor the response of vaccines. In comparison to H120, the test for HI using strain M41 as an antigen was not very efficient when used to identify antibodies after vaccination with H120 (de Wit *et al.* 1997).

1.6.4.2 Enzyme linked immunosorbent assay (ELISA)

ELISA is a convenient and sensitive method for field or experiment use in monitoring IBV antibodies after vaccination or infection (de Wit *et al.* 1997). Several commercial ELISA kits are available for detection of IBV-specific antibodies. IBV antibodies can be detected by ELISA within one week after vaccination or infection (de Wit *et al.* 1998; de Wit *et al.* 1997; Hawkes *et al.* 1983; Marquardt *et al.* 1981; Mockett & Darbyshire 1981). Due to the short period between infection and the production of the first antibodies detectable by ELISA, paired serum sampling is required: one at the first sign of infection, which is typically 18 to 36 hpi (Cavanagh & Naqi 2003) and a second sample seven days later. If the first sample is not taken early after infection, seroconversion may be missed (de Wit 2000; Jackwood & de Wit 2013). Indirect ELISAs have been investigated to produce serotype-specific ELISAs using serotype-specific monoclonal antibody (mAb) as a blocking agent (Garcia & Bankowski 1981; Karaca & Naqi 1993; Marquardt et al. 1981; Zellen & Thorsen 1987). Antibody titres can be first detected by ELISA followed by HI (de Wit *et al.* 1997), and last by virus neutralisation test (Marquardt et al. 1981; Mockett & Darbyshire 1981).

1.7 Vaccination

The prevention of IBV in chickens is achieved by the use of live and inactivated vaccines, which provide protection against losses caused by virulent field IBV viruses in broilers, commercial egg layers and breeders (Cavanagh & Naqi 2003; Jackwood & de Wit 2013). Live attenuated IB vaccines are the first generation IBV vaccines which have been attenuated by successive passage in SPF ECE (Klieve & Cumming 1988). Despite these preventive controls, an outbreak of IBV may still occur due to the emergence of novel IBV field strains in many parts of the world (Sjaak de Wit *et al.* 2011), including the persistence of some antigenic variants that makes prevention of IBV infections very challenging (Awad *et al.* 2016b; Gelb *et al.* 1991).

Vaccine strains are selected to represent the antigenic spectrum of isolates in a particular country or region (Dhama *et al.* 2005). Most commonly, live vaccines derived from virulent strains such as Massachusetts (Mass) M41 and the Dutch H52 and H120 strains (Jackwood & de Wit 2013; Lee et al. 2010). In order to obtain optimal protection, the live vaccine should contain serotypes of the virus which will stimulate protection against those existing/circulating strains in a particular area. For instance, in the USA, M41, H120, Connecticut types, GA98 and GA08 are used widely, whereas other serotypes such as Florida, Arkansas, JMK, and Cal99 are used regionally (Alvarado *et al.* 2003; Cook *et al.* 2012; Gelb *et al.* 1989; Jackwood *et al.* 2003; Martin *et al.* 2007). In Australia, strains of their B and C subtypes are used, and in the Netherlands, strains Holland- D274 and D1466 are used (Klieve & Cumming 1988; Wadey & Faragher 1981). Vaccine strains that include M41, 793B (4/91 and CR88), and D274 are used in European countries (Cook *et al.* 1999; de Wit *et al.* 2011a; Terregino *et al.* 2008). In

Asia, in addition to M41, 793B (4/91 and CR88), and D274, local strain vaccines have been used (Lin et al. 2005).

Live IB vaccines are used in broiler chicks and for the primary vaccination in breeders and layers (Klieve & Cumming 1988). Live attenuated vaccines may be applied to day-old broilers individually by the oculonasal route (Cook et al. 1999). However, in the field, live vaccines are usually administered via drinking water, by coarse spray, or aerosols , either at one day-old or within the first week post hatch (Jackwood & de Wit 2013). Although these methods of mass administration are popular because of their convenience, there may be problems in achieving uniform application of the vaccine, and more severe respiratory reactions may be caused by the aerosol method (Saif *et al.* 2003). It is possible that post-vaccination respiratory reactions might directly affect bird health and productivity. Therefore, gel vaccination was suggested as a new method for IBV control and vaccine administration. By this method, negligible respiratory signs were detected following to vaccination with combined strains (793B 1/96 and Mass B-48) (Tucciarone et al. 2018).

As the duration of immunity following live vaccine administration is short, a booster vaccination is administered with the same strain, or a combination of other strains, 2-3 weeks after primary application (Cavanagh 2003). The benefit of administering two serologically different vaccines is a broad immunity against heterologous strains (Awad *et al.* 2015; Ball *et al.* 2017; Chhabra *et al.* 2015b; Cook *et al.* 2001; Cook *et al.* 1999; Terregino *et al.* 2008). Moreover, this vaccination approach was discovered to be more efficient than revaccination with a vaccine of the same serotype as the initial vaccine (Cook et al. 1999).

Inactivated oil emulsion or killed vaccines are used primarily at the point of lay in breeders and layers (Box et al. 1988). These has been used either alone or in combination with live attenuated IBV vaccines to obtain long-lasting immunity before the onset of egg production (Cavanagh 2003; Finney *et al.* 1990). These vaccines are usually administered by the intramuscular or subcutaneous route at 13-18 weeks of age, for the induction of high levels of humoral antibodies which can protect the oviduct against circulating IBV and avoid decreased egg production or quality. Whole killed IBV can protect chickens from virulent challenges (Cavanagh & Naqi 2003). However, it has been reported that although the parenteral inoculation of inactivated IBV vaccines limits the incidence of nephrosis, it does not adequately stimulate the immune response necessary to protect the respiratory tract (Cavanagh et al. 1984; Ignjatovic & Galli 1994). Compared to live vaccines, inactivated vaccines induce a short-term immune response, which is characterised by humoral antibody production but not T-cell-mediated responses (Collisson et al. 2000; Ladman et al. 2002). Therefore, the efficacy of killed vaccines, in most cases, depends on correct priming with live vaccines (Cavanagh 2003; Jackwood & de Wit 2013). Immunisation of young females with live vaccines on more than one occasion, followed by a single dose of killed vaccine before the birds come into lay, is a common practice on poultry farms.

New vaccination methods, such as plasmid DNA vaccines, subunit vaccines, vector-based vaccines and reverse genetic vaccines, have been made possible with advances in molecular biology methods. Experimental recombinant vector vaccines which most commonly contain the IBV S1 glycoprotein have been developed against IBV. It has been shown that these vaccines were able to induce immune responses and protect against tracheal lesions following homologous and heterologous challenges with Vic S

(serotype B) or N1/62 (serotype C) IBV strains (Johnson et al. 2003). While the advances in viral vector vaccines seem promising in providing effective immune response against IBV, this technology does have some limitations, which include the issue of pre-existing immunity or maternally derived immunity to the live vector itself, which reduces the uptake of the antigen by the antigen-presenting cells (Faulkner et al. 2013). However, oral immunisation of mice with an adenovirus vector has been shown to avoid neutralisation of the vector by maternally derived antibodies (Xiang et al. 2003).

A DNA vaccine based on the S1-gene of the Arkansas IBV serotype was developed (Kapczynski et al. 2003). Vaccination via the *in ovo* route, followed by application of a live attenuated vaccine at 2-week intervals, resulted in a significant immune response and 100% protection against clinical disease, compared to live virus vaccine or *in ovo* DNA vaccination only (Kapczynski et al. 2003).

Recently, using reverse genetics technology, a recombinant BeauR-IBV vaccine has been constructed by substituting the antigenic S1-glycoprotein of an apathogenic Beaudette-IBV strain with another S1-gene, by expressing the full S ectodomain from 4/91 (Armesto et al. 2011), or by expressing the M41 ectodomain (Casais et al 2005). In another study, a modified H120 (R-H120) virus was constructed and found to elicit a high level of HI antibodies and a protection rate comparable with an intact H120-vaccinated group (Zhou et al. 2013). These recombinant vaccines can be administered in *ovo*, but whether they will impact the rate of mutation and viral selection pressure, or prove to be more economical than existing traditional vaccines is yet to be determined. Hence, further consideration should be given to the use of new-

generation vaccines as an alternative to conventional vaccines, to generate protective immunity against IBV.

1.8 Aims of the thesis

1. To study the broiler chicken innate immune responses in the head associated lymphoid (HALT) and respiratory tissues after virulent IBV M41 infection in commercial broiler chickens (Chapter 3).
2. To explore the broiler chicken immune responses in the HALT and respiratory tissues for vaccinated-unchallenged and vaccinated-challenged chicks (Chapter 4).
3. To compare the broiler chicken immune response following IBV vaccination by different methods of vaccination - Intranasal, Spray or Gel (Chapter 5).
4. To examine the layer chicken hen immune responses following homologous (Mass or 793B) IBV vaccination by drinking water or oculonasal routes (Chapter 6).

Chapter 2: Materials and methods

2.1 Specific-pathogen-free (SPF) eggs

All SPF embryonated chicken eggs (ECE) that were used for preparation of tracheal organ cultures (TOCs), virus titration, and propagation in all chapters were obtained from a commercial source[1]. Eggs were incubated at 37 °C with 55% humidity. The parental flock was free from chicken disease agents, including IBV.

2.2 Tracheal organ culture (TOC)

All TOCs were prepared at 18-19 days post incubation, under sterile conditions. Eggs were wiped with 70% alcohol, viable embryos were humanely euthanised, and sterile scissors and forceps were used to remove tracheas. The trachea was placed in pre-warmed TOC culture medium (containing Eagle's serum-free minimum essential medium (MEM) with glutamine, and 10x concentration of streptomycin (250 mg/ml) and penicillin (150 mg/ml). Streptomycin/ Penicillin solution 4 ml was added to 100 ml of medium (Ganapathy 2009).

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 14 ml round-bottom tube[3] containing 600 µl of pre-warmed TOC media. Tubes were then incubated in a rotating incubator[4] (8 rpm) at 37 °C. Ciliary activity was checked after 24 hours using an inverted microscope under low power (100x magnification). Only rings with 100% ciliary activity were used for all experiments.

[1] Novartis, Liverpool, UK

[2] Campden Instruments Ltd, Loughborough, Leicestershire, UK

[3] Thermo Scientific, Warrington, UK

[4] Lab Thermal Equipment, Greenfield, nr. Oldham, UK

2.3 Broiler chicks and layer hens for *in vivo* studies

Day-old broiler Ross 308 chicks with IBV maternally-derived antibodies (MDA) were purchased from a commercial hatchery[5] (Figure 2.1 A). Forty-one weeks-old light brown Lohman layer chickens were purchased from a commercial farm (Figure 2.1 B).

Birds 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) provided *ad-libitum* (Figure 2.2). All experimental procedures were undertaken following approval by the University of Liverpool ethical review committee under the project license PPL 40/3723 and according to UK legislation on the use of animals for experiments.

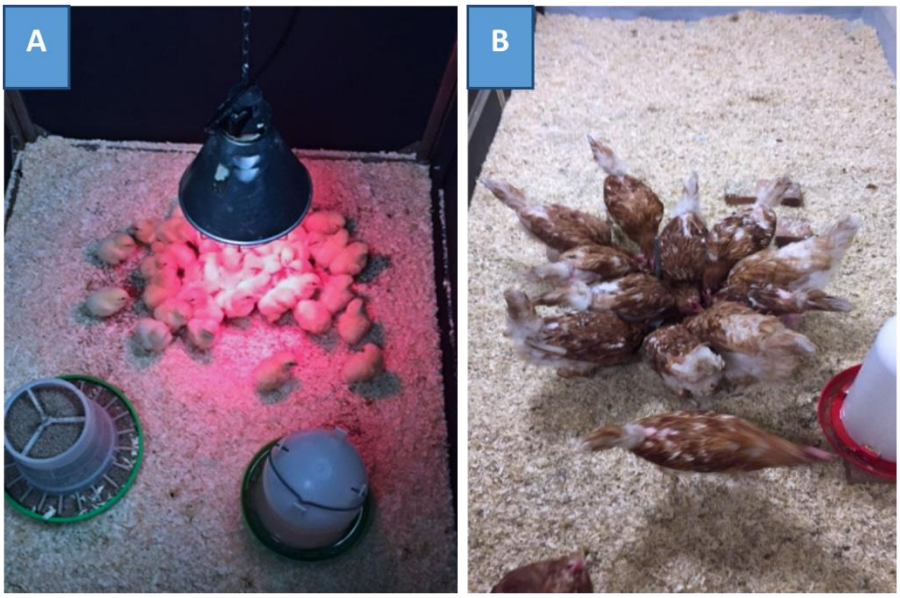

Figure 2.1. (A) One day old broiler chicks and (B) 41 weeks-old light brown Lohman layer hens for *in vivo* experiments.

[5] Frodsham Hatchery, Frodsham, UK

2.4 Chick welfare and management

All birds were checked twice daily to ensure their health and welfare. Chicks were housed securely with ventilation and temperature adjusted according to age. Individual groups were kept in separate isolation rooms to prevent cross-contamination. These included footbaths with 2% Virkon[6] disinfectant placed both inside and outside each experimental room. Personal protection equipment (PPE) was used at all times during post-mortem dissection and tissue collection, including disposable facemasks, gowns and gloves.

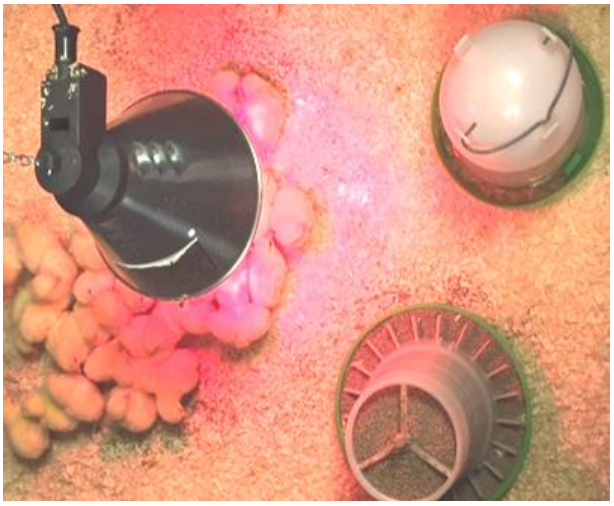

Figure 2.2. Chicks at day one old rearing in deep litter and chicks were given *ad libitum* access to food and water.

2.5 IBV vaccines for *in vivo* experiments

All vaccines were kept at 4 °C until used. Details of individual vaccines used per experiment are given in the respective chapters.

[6] Antec International, Suffolk, UK

2.6 Propagation of virulent infectious bronchitis viruses (IBVs) in ECE

Viral titration was performed using 10 days old SPF ECE via the allantoic cavity. At candling, the air cell was marked to avoid the area with blood vessels. The marked area was disinfected with 70% alcohol and a 1 ml syringe with a 25 gauge needle was used to inoculate eggs. The holes were sealed and eggs were incubated at 37 °C in an incubator for 48 hours. Eggs were examined daily by candling, and any embryos which had died were discarded.

Allantoic fluid containing infectious bronchitis viruses was then harvested, centrifuged at 700 × g for 5 mins to clarify supernatant, and 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 (Awad 2014). All IBV-rich allantoic fluid was titrated in TOCs according to the Reed-Muench method (Reed & Muench 1938), aliquoted and stored at -70 °C.

2.7 Ciliostasis test for *in vivo* studies

Following euthanasia, tracheas were extracted during necropsy to determine the level of vaccine protection in the trachea. Each trachea was cut (3 rings from the proximal end, 4 rings from the middle and 3 rings from the distal end) using a tissue chopper[2] and placed in a petri dish[7] containing warm TOC medium and examined under low (100x) magnification. Ciliary protection scoring was recorded as described previously: 100% cilia beating = 0; 75% cilia beating = 1; 50% cilia beating = 2; 25% cilia beating = 3; no beating (complete ciliostasis) = 4 (Awad *et al.* 2016c). The mean ciliary score for

[7] Sterilin Ltd, Hounslow, UK

each bird was used to calculate percentage protection for each group (Awad *et al.* 2016c; Cook *et al.* 1999). According to the European Pharmacopoeia's reference standards, vaccinated chickens yielding 50% or more tracheal explants with ciliary activity were considered to have been protected against the challenge virus (Council of Europe 2007; de Wit & Cook 2014). Protection scores were calculated according to the following formula:

$$\text{Protection Score} = \left(1 - \frac{(\text{Mean score for vaccinated/Number birds challenged group })}{(\text{Mean score for unvaccinated/Number birds unchallenged })}\right) * 100$$

2.8 Swab, blood and lachrymal fluid sampling

2.8.1 Swab

Dry swabs[8] were used to sample the oropharyngeal tissue and the cloacal cavity. After sampling, swabs from each group were pooled and dipped into 1.5 ml of TOC media or guanidine thiocyanate (solution D). The media underwent 2x freeze-thawing and then stored at -70 °C until required for RNA extraction.

2.8.2 Blood

Blood was collected from the brachial wing vein using a 23 gauge sterile needle[9] and 2 ml syringe[9], and placed in labelled bijou tubes without coagulant. In order to obtain serum separation, tubes were kept overnight at room temperature in a slanted position. Next day, samples were centrifuged at 3,000 g for 10 mins, sera collected and stored at -20 °C.

[8] Medical Wire and Equipment (MWE), Corsham, UK

[9] Becton, Dickinson and Co. Ltd

2.8.3 Lachrymal fluid

Approximately 0.003 g fine sodium chloride crystals were sprinkled onto one eye while keeping the eyelids held open, and within one-minute lachrymal fluids were carefully collected by a pipette. The fluid was immediately placed in 1.5 ml Eppendorf tubes[10] and immediately centrifuged at 3000 x g for 5 mins before the supernatant was stored at -20 °C as previously described (Ganapathy *et al.* 2005; Dhinakar Raj & Jones, 1996).

2.9 Clinical signs and gross lesion assessments

2.9.1 Clinical signs

Clinical signs were recorded daily according to a previously published scoring system (Grgic et al. 2008) (Table 2.1), using the following criteria: coughing, râles or depression were considered as mild signs (score = 1); moderate signs considered as depression, along with gasping or ruffled feathers were moderate signs (score = 2); whereas severe gasping (mouth breathing), coughing, sneezing or depression accompanied by ruffled feathers were recorded as severe signs (score =3). Absence of any clinical signs was scored as 0.

Table 2.1. Scoring system used for clinical signs assessment in chickens following M41 IBV infection.

Signs	Score
No clinical signs	0
Coughing, râles or depression	1
Depression, along with gasping or ruffled feathers	2
Severe gasping, coughing, sneezing or depression accompanied by ruffled feathers	3

2.9.2 Gross lesions

Tracheal tissues were observed following culling and dissection, with gross lesions recorded as described below (Mahgoub et al. 2010) (Table 2.2).

Table 2.2. Scoring system used for gross lesion assessment in trachea following M41 IBV infection.

Lesions	Score
No lesion	0
Congestion	1
Mucoid exudate	2
Caseous plug, or in case of airsacculitis, perihepatitis and pericarditis	3

2.10 Tissue samples for quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Tissue samples were collected from euthanised birds using sterile instruments and placed in labelled bijous or 1.5 ml Eppendorf tubes[10] containing RNA later[11] at -20 °C.

2.11 Histopathology

Tracheal tissues were fixed in 10% buffered formalin immediately after necropsy and kept at room temperature (20-25 °C) for 24-48 hours, processed for histological examination, then embedded in paraffin and sectioned at 5 µm thickness 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 for one minute followed by a final wash with 95% and then 100% ethanol. Slides were washed in xylene, and

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

[11] Qiagen, Manchester, UK

coverslipped. Samples were examined by a veterinary pathologist at the University of Liverpool. Histological changes in each bird were scored as follows: 0 = no change, 1= mild, 2 = moderate, 3 = severe, for each of the following categories: loss of cilia, epithelial degeneration, decrease in mucous cells, heterophil infiltration, epithelial hyperplasia and lymphoid infiltration (Chen & Itakura 1996; Chhabra 2016a). The total score for each bird was used to calculate the mean score ± SEM for the group. Numbers of tracheal processing were different due to low availability for tracheal parts as the priority was the cilia-stopping test, viral RNA load, mRNA expressions and immunohistochemistry.

2.12 RNA extraction methods from swabs for RT-PCR

2.12.1 Extraction of RNA using phenol-chloroform method

The phenol-chloroform method was used as previously described (Ball et al. 2016a; Chomczynski & Sacchi 2006). Briefly, 300 µl of TOC media from swabs was treated with 300 µl of solution D and kept at -20 °C overnight. This was then mixed with 50 µl sodium acetate and 650 µl phenol:chloroform 5:1 and then centrifuged at 13,000 g for 5 mins. The top layer was removed and mixed with 500 µl isopropanol and then left overnight at -20 °C to precipitate. Following this, the sample was centrifuged at 13,000 g for 15 mins. 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 (Dithiothreitol) and 92.5 µl water) and left to stand at 4 °C for at least 15 mins. Samples were then vortexed for 10 seconds before proceeding for RT-PCR (Chomczynski & Sacchi 2006).

2.12.2 Extraction of RNA using a commercial kit

Viral RNA was extracted using the QIAamp viral RNA Mini Kit[12] according to the manufacturer's instructions. In brief, 140 µl of each sample was added to a 1.5 ml Eppendorf tube[10] containing 560 µl of AVL buffer plus carrier RNA, and vortexed for 15 seconds. Samples were incubated at room temperature for 10 mins and pulse centrifuged. Following this, 560 µl 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 and centrifuged at 8,000 rpm for 1 minute; flow through was discarded and this step was repeated with the remaining sample. The spin column was placed in a clean collection tube along with 500 µl of buffer AW1 and centrifuged at 8,000 rpm for 1 minute and flow through was discarded. The previous step was repeated using AW2 buffer. Spin columns were centrifuged at 13,000 rpm for 3 mins and the flow through was discarded. Centrifugation was repeated at 13,000 rpm for 1 minute. As a final step, 60 µl of Sigma water was used to elute the RNA from the column and stored at -20 °C.

2.13 Total RNA extraction from tissues stored in RNA later®

Total RNA extraction was carried out using the RNeasy Mini Plus kit[13] for tissue samples. From each tissue, 30 mg was weighed out and were cut into small pieces using sterile scissors in 2 ml Eppendorf tube[14] and mixed with 600 µl of RLT buffer with mercaptoethanol (10µl mercaptoethanol in 1 ml RLT buffer). Tissues were homogenised using a Tissue Lyser[15] (Bullet Blender Storm) and a 5 mm stainless steel

[12] Qiagen, Manchester, UK

[13] Qiagen, Manchester, UK

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

[15] Storm Bullet Blender, USA

bead, and the lysate was centrifuged at 13000 rpm for 3 mins. The solution (550 µl) was transferred to a spin gDNA eliminator spin column. Samples were then centrifuged for 30 sec at 10,000 rpm and flow through was saved in the collection tubes. To 550 µl of the supernatant, a similar volume of 70% ethanol was added and 700 µl including any precipitate was placed in an RNeasy spin column. Washing and elution were performed according to the manufacturer's instructions using RW1 and RPE buffers in two stages. As a final step, 35 µl RNase-free water was used to elute the RNA from the column.

A NanoDrop[16] 1000 spectrophotometer was used to quantify the extracted RNA yield (ng/µl). The extracted RNA was stored at -70 °C until used for quantitative reverse transcription PCR (qRT-PCR) analysis and preparation of cDNA for host gene and cytokine mRNA expression.

2.14 RT-PCR protocol

The IBV RT-PCR was conducted as previously described (Ball *et al.* 2016c; Cavanagh *et al.* 1999; Ganapathy *et al.* 2015; Worthington *et al.* 2008) that targets a 393bp portion of the S1 gene.

2.14.1 Reverse transcriptase (RT)

The RT reaction mix comprised of 5x Buffer, DTT, dNTPs, Rnase inhibitor, Superscript II RT[17], water and SX2- primer (Table 2.3). Five microliters were added into a labelled 0.2 ml Eppendorf tube for each sample, along with 0.5 µl of extracted RNA. The tubes were

[16] Thermo Scientific, Wilmington, DE

[17] Invitrogen, Life Technologies, Inchinnan, Scotland

then placed in a thermocycler[18] and run under the following conditions: 42 °C for 1 hour, 72 °C for 10 mins and then held indefinitely at 8 °C.

2.14.2 Nested PCR 1

A master mix containing DNA polymerase, magnesium chloride ($MgCl_2$), dNTPs and buffer was prepared and 19 µl added to the product of reverse transcriptase, with 0.5 µl of each primer (SX1+ and SX2-; Table 2.3)[19]. Samples were run under the following conditions: 94 °C for 15 seconds followed by 35 cycles of 94 °C for 10 seconds, 50 °C for 20 seconds, and 72 °C for 40 seconds and then held indefinitely at 8 °C.

2.14.3 Nested PCR 2

The master mix was prepared as follows; 24µl Supermix mixed with 0.5µl- oligo SX3+ and 0.5µl oligo SX4-. After vortexing, 24.5 µl from the total volume of the reaction mixture was placed into 0.2 ml clip top Eppendorf tube. Then, 0.5 µl of PCR 1 product was added. Samples were placed in a thermocycler under the following conditions: 94 °C for 15 seconds for 1 cycle and 35 cycles at 94°C for 10 seconds, 50 °C for 20 seconds and finally 72 °C for 40 seconds. Finally, samples were held at 8 °C.

[18] GeneAmp PCR system 9700, Applied Biosystems, Warrington, North West England, England

[19] MWG Eurofins, Edersberg, Germany

Table 2.3. Oligonucleotides used in RT- PCR and reverse transcriptase amplification.

Step	Oligonucleotides	Sequence (5' to 3')	Gene	Product size (bp)	Position in gene (bp)
Nested PCR 1	SX1+	CACCTAGAGGTTTG T/C T A/T GCAT			677-698
	SX2-	TCCACCTCTATAAACACC C/T TT	S1	393	1148 -1168
Nested PCR 2	SX3+	TAATACTGG C/T AATTTTTCAGA			705 - 725
	SX4-	AATACAGATTGCTTACAACCACC			1075-1097

2.14.4 Agarose gel electrophoresis

A 1.5% w/v agarose gel was prepared by dissolving agarose in 1x TBE buffer (Tris-borate-EDTA) by heating in a microwave. After cooling, a nucleic acid solution (Redsafe^{TM})[20] was used to stain the agarose. The agarose was poured into a mould (12 x 9 cm) Hybaid Electro-4 gel tank[21] consisting of 20 wells. After 15-20 min 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. A 100 bp ladder (molecular marker[22]) was included for comparing the amplicon size. The gel was ran at 75V for 50 mins in 1x TBE buffer, and then viewed and analysed using an ultraviolet transilluminator[23].

[20] Intron Biotechnology, Inc, Sangdaewon-Dong, Korea

[21] Hybaid Ltd, Middlesex, UK

[22] Amersham Pharmacia Biotech, Buckinghamshire, UK

[23] UVitec Ce Mo32977 transiliminuim UVITEC celt

2.15 Immunohistochemistry

2.15.1 Cryostat (tissue section)

Collected samples were stored in optimal cutting temperature (OCT)[24] embedding compound. Samples were immediately frozen after dissection in -190 °C liquid nitrogen and kept at -70 °C. Samples were cut at 5-8 µm thickness and mounted on a microscopic glass slide[24] using a pre-cooled cryostat[25], covered with a glass slide and kept in the dark overnight. Fixation was carried out with pre-cooled acetone (- 20 °C) for 10 mins. Finally, samples were dried at room temperature for around 10 mins and stored at -70 °C until staining (Bezuidenhout *et al.* 2011; Chhabra *et al.* 2015b).

2.15.2 Immunohistochemistry (IHC) staining

Slides were air dried at room temperature for 10 mins before staining. To prevent solutions running off the slides an ImmEdge Hydrophobic Barrier PAP Pen[26] was used to draw a barrier around the sections, and the sections were washed in Tris Buffered Saline (TBS) for 5 mins. To reduce endogenous peroxidase activity to prevent cell lysis, sections were treated with 0.03% hydrogen peroxide (H_2O_2) in PBS for 10 mins. Subsequently, blocking serum from Vectastain Elite ABC kit[27] was applied to tissue sections for 10 mins to prevent non-specific binding. Sections were incubated overnight at 4 °C in the dark in a humidified chamber with anti-IBV nucleoprotein

[24] Solmedia laboratory, Shrewsbury, UK

[25] LEICA CM 1900, Nussloch, Germany

[26] Vector Laboratories, Burlingame, UK

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

monoclonal primary antibody (mouse monoclonal antibody[28]) diluted at 1:20,000 in the commercial antibody diluent[29].

A biotinylated anti-mouse IgG was used to detect the primary antibody[27]. Sections were incubated at room temperature for 30 mins followed by a 4% 3, 3-diaminobenzidine solution (DAB) containing 2% H_2O_2. This was followed by a 2% buffer stock solution for colour development at room temperature for 5 mins. Sections with no primary antibody were used as a negative control (Abdel-Moneim et al. 2009). Slides were washed in dH_2O counterstained in haematoxylin for 1 minute, and "blued" in running tap water for 5 mins. Finally, slides were dehydrated (once with 96% ethanol for 1 minute; two washes in 100% ethanol for 3 mins) before clearing in xylene and cover slipping with Distyrene Plasticizer Xylene (DPX). IBV-positive cells were observed and counted in three sites in each tissue section under the microscope fields at 400x magnification, and scored as none (0) for less than 5, mild (1) for 5 to 50, moderate (2) for 50 to 150 and severe (3) for over 150 positively-stained cells (Oladele et al. 2009). The mean score for the three fields was expressed as the antigen score.

2.16 Partial purification of IBV-M41

IBV M41 was propagated in in 10-day-old ECEs. Partial purification was done as previously described (Ganapathy *et al.* 2005; Keep *et al.* 2015) by adding 25 ml of IBV-rich allantoic fluid into a 50 ml universal tube and centrifuged for 10 mins at 1,150 × g (4 °C). The pellet was washed, re-suspended in phosphate-buffered saline (PBS) and filtered through a 0.22 µm filter. Then, 10 ml of 30% sucrose was added to an

[28] Prionics, Lelystad, The Netherlands

[29] Dako EnVension Flex, Glostrup, Denmark

ultracentrifuge tube[30] and the virus suspension was overlaid and centrifuged for 4 hours at 102400 × g (4 °C) in an ultracentrifuge[31]. The supernatant was carefully removed and discarded. The pellet was then washed twice and re-suspended in PBS (volume of PBS depends on pellet size; 100µl or 200µl), aliquoted and stored at -70 °C.

2.17 Protein quantification using the Bradford assay

Total protein was quantified using the 5x Bradford assay[32]. In brief, serial dilutions of bovine serum albumin[33] (BSA) was used for standard curve preparation (Table 2.4). Unknown samples were diluted in two ratios of 1:20 and 1:40 in PBS. For the negative control, 50 µl distilled H_2O was added to a 96-well microplate[34]. Then, 50 µl of BSA dilution and the unknown samples (1:20 and 1:40) were transferred to wells. Following this, 200 µl of diluted Bradford reagent was added to all wells. All reagent and samples were tested in triplicate. Plates were incubated for 5 mins at room temperature then the optical density (OD) was read at 595nm using the Multiskan FC[35]. The purified antigen protein concentration was obtained after comparing obtained OD to the BSA standard curve.

[30] Beckman Coulter, Ultra-clear tube (25X89mm), Indiana, U.S.A

[31] Beckman Coulter, Optima XPN-80, Indiana, U.S.A

[32] SERVA Electrophoresis GmbH, Heidelberg, Germany

[33] Sigma Aldrich®, Dorset, UK

[34] Flat bottom 96-well microplate, STRA LAB, UK

[35] Thermo Fisher Scientific, Warrington, UK

Table 2.4. The reference protein dilution (0, 20, 30, 40, 50, 60, 80 and 100 µg/ml to create a calibration curve. The assay is performed as triplicate determination.

BSA Concentration (µg/ml]	Volume (µl) of BSA Stock (100µg/ml)	Volume of PBS diluent (µl)
100	200	0
80	160	40
60	120	80
50	100	100
40	80	120
30	60	140
20	40	160
0	0	200

2.18 Serology

2.18.1 Enzyme-linked immunosorbent assay (ELISA)

Serum samples were analysed using a commercial IBV ELISA kit[36] according to the manufacturer's instructions. Sera were diluted 1:500 with the supplied diluent and added to coated antigen plates. Plates were incubated for 30 mins at 25 °C then washed 3-5 times with 350 ml of sterile distilled water. Following this, 100 µl conjugate was added then incubated for a further 30 mins. The wash step was repeated, then substrate was added and further incubated away from direct light. Finally, 100 µl of stop solution was added to each well. Plates were read using a microplate reader Multiskan® FC[35] at 650 nm to determine the sample absorbance. Samples with a sample/positive (S/P) ratio greater than 0.2 were considered as positive. The titre calculation was achieved using the following formula:

[36] IDEXX, Westbrook, Maine, U.S.A

$$\text{Titre} = log10 + 1.09(\log S/P) + 3.36$$

2.18.2 Local antibody detection from lachrymal fluids using indirect monoclonal ELISA

Lachrymal fluid samples were collected and assayed for the detection of IBV specific IgA and IgY using an indirect ELISA (Mockett & Cook 1986; Raj & Jones 1996b). In brief, each well of flat bottom 96-well microplate[34] was coated with 100 µl of 2.5 µg/ml M41 antigen in 50 mM sodium carbonate/bicarbonate coating buffer (CBC) (pH 9.6). 100 µl CBC was added to two wells without antigen as a negative control. The plate was covered with a polyethylene adhesive seal[37] and incubated for 1 hour at 37 °C, and then overnight at 4 °C. The plate was washed 3-5 times with PBS containing 0.05% Tween-20 (PBST)[33]. Wells were blocked with 200 µl per well phosphate buffer saline (PBS) containing 3.0% non-fat skimmed milk powder and the plate incubated for 1 hour at 37 °C, or overnight at 4 °C. This was then washed once with PBST[33]. Lachrymal fluid samples were tested in triplicate at a dilution of 1:10 in PBST[33]. The plate was covered and inculpated for 1 hour at 37 °C then washed 3-5 times with PBST.

Following this, mouse monoclonal antibodies against either chicken IgA[38] or IgY[38] at a dilution of 1:2,500 and 1:1,000 respectively were added (50 µl) as the secondary antibody, and the plate was covered and incubated for 1 hour at 37 °C. The wash step was repeated followed application of 50 µl goat anti-mouse IgG horse- radish peroxidase-conjugate[38] at a dilution of 1:10,000. The plate was covered and incubated

[37] STAR LAB, Milton Keynes, UK

[38] BIO-RAD®, Hertfordshire, UK

for 1 hour at 37 °C. The plate was washed as above and the tetramethylbenzidine (TMP)[33] substrate was added to each well (50 µl) and incubated in the dark for 15-20 mins until colour development was complete. The reaction was stopped by adding 50 µl of sodium hydrochloric acid (0.5 M HCl), and the plates were analysed at 450 nm using a microplate reader Multiskan® FC[35]. Corrected optical density (COD) values were calculated by deducting the OD values of non-antigen coated (blank) wells from those of the test wells (Fournier-Caruana *et al.* 2003; Ganapathy *et al.* 2005).

2.19 cDNA synthesis

The SuperScript III First-strand synthesis system[39] was used to prepare first strand cDNA from the total RNA extraction. The reaction mixture was prepared from random primers, diethyl-pyro carbonate (DEPC) treated water and dNTP mix[39], and 1 µg RNA added. The sample was incubated for 5 mins at 65 °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 5000 rpm for 10 s, followed by incubation at 25 °C for 10 mins, then 50 °C for 50 mins and the reaction was terminated at 85 °C for 5 mins.

2.20 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 uses the IBVRT1 forward primer CTATCGCCAGGGAAATGTC, and the IBVRT2 reverse primer GCGTCCTAGTGCTGTACCC, along with the IBVRT3 TaqMan® probe FAM-

[39] Invitrogen, Life Technologies, Inchinnan, Scotland

CCTGGAAACGAACGGTAGACCCT-TAMRA. The qRT-PCR Rotor® gene® probe, Real-Time PCR kit[40] (qPCR) (one-step) was utilised according to manufacturer's instructions using 40 ng of total RNA per reaction.

Cycling conditions were 10 mins at 50 °C and 5 mins at 95 °C followed by 40 cycles of 5 s at 95 °C, 10 s at 60 °C (annealing temperature) and 10 s at 72 °C. The threshold cycle (Ct) was measured, amplification plots were recorded and analysed using Rotor-Gene® Q thermocycler software. Obtained Ct values were converted to log relative equivalent units (REU) of viral RNA by a standard curve generated from using five 10-fold dilutions of RNA extracted from M41 virus-positive allantoic fluid (Londt et al., 2013).

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

Host gene expression was evaluated using cDNA synthesised as described previously. The relative expression was calculated using qRT-PCR and each sample was analysed in triplicate. The LightCycler® 480[41] was used to quantify the selected genes[42] (Table 2.5). 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. The cDNA was diluted in sigma water at a ratio of 1:50 and was added to 15 µl of the prepared master mix for a total volume of 20 µl. The cycling conditions were 10 mins at 95 °C, followed by 45 cycles of 10 s at 95 °C, 10 s at 60 °C and 10 s at 72 °C. A melt curve analysis was conducted to ensure the specificity of the assay. Data obtained from the qRT-PCR were normalised against 18S ribosomal RNA expression (Chhabra *et al.* 2016; Kuchipudi *et*

[40] Qiagen, Manchester, UK

[41] Roche, Welwyn Garden City, UK

[42] MWG Eurofins, Edersberg, Germany

al. 2012; Okino *et al.* 2013), and presented as fold change of gene expression compared to the control group.

Table 2.5. Primers used in the qRT-PCR analysis of gene signatures, interferons and cytokines.

Gene Group	Gene Target	Primer sequences: Forward (F) and reverse (R)	Reference
1. Reference gene	18S ribosomal RNA	(F) TGTGCCGCTAGAGGTGAAATT (R) TGGCAAATGCTTTCGCTTT	(Chhabra et al. 2018; Kuchipudi et al. 2012; Okino et al. 2013)
2. Viral recognition	TLR3 (Toll like receptor 3)	(F) GCAATTTCTCCTTCACCTTTTCA (R) CCTTTATGTTTGCTATGTTGTTATTGCT	(Kuchipudi et al. 2014)
	MDA5 (Melanoma differentiation-associated protein 5)	(F) AGGAGGACGACCACGATCTCT (R) CCACCTGTCTGGTCTGCATGT	(Kuchipudi et al. 2014)
3. Interferons	IIFN-α (Interferon alpha (Type I))	(F) CTTCCTCCAAGACAACGATTACAG (R) AGGAACCAGGCACGAGCTT	(Kuchipudi et al. 2014)
	IFN-β (Interferon beta (Type I))	(F) TCCAACACCTCTTCAACATGCT (R) TGGCGTGTGCGGTCAAT	(Kuchipudi et al. 2014)
4. Inflammation	IL-6 (Interleukin 6)	(F) CACGATCCGGCAGATGGT (R) TGGGCGGCCGAGTCT	(Kuchipudi et al. 2014)

2.22 Statistical analysis

All statistical analysis was done using GraphPad Prism version 6. Data were analysed according to data type obtained (Lopes et al. 2014). Parametric data (normally distributed) were 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 for two groups. Differences between groups were considered significant at $P<0.05$.

Chapter 3: Immune responses in head-associated lymphoid and respiratory tissues following IBV M41 infection in naïve commercial broiler chicks

Abstract

Infectious bronchitis virus (IBV) is a highly contagious pathogen that causes respiratory, renal and reproductive diseases in chickens. The virus primarily replicates in the epithelial tissues of the respiratory tract, such as the choanal cleft, turbinate, pharyngeal tissues and trachea. Little information is available on immune responses in such head- associated lymphoid (HALT) and respiratory tissues following IBV infection. For this reason, we examined the viral load, pro-inflammatory cytokines and host gene signatures in the HALT and respiratory tissues of chickens experimentally challenged with IBV M41. Twenty-one-day-old commercial broiler chickens were divided into two groups and challenged via the oculonasal route as follows: (i) group one was challenged with virulent IBV M41 (0.1 ml of $10^{5.75}$ $TCID_{50}$/chick) and (ii) group two was mock-challenged with 0.1 ml of virus-free allantoic fluid. At 1-5 dpc, lachrymal fluid was collected for anti-IBV IgA and IgY using monoclonal ELISA. Harderian gland (HG), choanal cleft, turbinate, pharyngeal tissue and trachea tissues were collected. RNA was extracted for virus quantification, pro-inflammatory cytokine (IL-6) and host gene mRNA expression, including TLR3, MDA5, IFN-α and IFN-β. The IgA levels were elevated at 4–5 dpc. At 5 dpc, the viral load in the turbinate and choanal cleft was higher than those found in the HG, pharyngeal and tracheal tissues. Immunohistochemistry (IHC) confirmed presence of IBV in all examined tissues with peak viral antigen score measured at 3 dpc. Data showed high levels of mRNA gene expression of TLR3, MDA5, IFN-α, IFN-β and IL-6 in tissues from challenged groups compared to the unchallenged control group. Our preliminary findings demonstrate an innate immune response at 1– 3 days after M41 challenge. More specifically, there was a marked up-regulation of TLR3, MDA5, IFN-β and IL-6 mRNA expression in the HALT and respiratory tissues.

3.1 Introduction

Infectious bronchitis virus (IBV) is a highly contagious pathogen that causes chickens to suffer from respiratory, renal and reproductive diseases. IBV replicates primarily in the respiratory tract's epithelial tissues, such as turbinate, choanal cleft, pharyngeal tissues and the trachea. The IBV is highly infectious and can spread through airborne transmission, faecal ingestion, and contaminated feed or water.

Over the years, serological and molecular studies have been extensively conducted to determine the epidemiology of local IBV strains (Awad *et al.* 2014b; de Wit *et al.* 2011a; Jackwood 2012). The Mass serotype was discovered in the 1930's and is one of the most widespread serotypes, it replicates primarily in the respiratory tract and subsequently emigrates and replicates in a range of other tissues (Alvarado *et al.* 2006; Jia *et al.* 2002).

Pattern recognition receptors (PRRs) are present on the mucosal surfaces of immune cells. Through PRRs, such as Toll-like receptors (TLRs), RIG-I like receptors (RLRs), Melanoma differentiation-associated protein 5 (MDA5) and NOD-like receptors (NLRs), these cells rapidly recognise infectious agents (Chhabra *et al.* 2015a). TLRs are one of the more prominent immune cell receptors, and an up-regulation of TLR2, TLR3, TLR6 and TLR7 mRNA expression has been reported in tracheal epithelial cells after attenuated IBV Massachusetts intranasal inoculation (Guo *et al.* 2008; Wang *et al.* 2006). The function of TLRs in viral immunology is well established (Le Goffic *et al.* 2007; Liu *et al.* 2007). MDA5 expression levels were reported to be significantly increased in chicken kidney tissue after nephropathogenic IBV infection, suggesting a role for MDA5-limiting IBV infection in chickens (Cong *et al.* 2013). Recent research has suggested that *in vitro* virulent IBV infection leads to a significant induction of IFN-β

transcription through an MDA5-dependent activation of the IFNs response (Kint *et al.* 2015).

Limited information is available on immune responses in head-associated lymphoid (HG and choanal cleft) and respiratory tissues (Pharyngeal tissue turbinate and trachea tissues) following IBV infection. For this reason, we examined the viral load, pro-inflammatory cytokines and host gene signatures in the HALT and respiratory tissues of chickens experimentally challenged with IBV M41.

3.2 Material and methods

3.2.1 Chicks

Sixty, day-old broiler chicks were obtained from a commercial hatchery and kept according to animal welfare guidelines, under strict biosecurity measures in an isolation unit at the University of Liverpool (Chapter 2.3). Chicks were reared in deep litter, with antibiotic-free water and feed provided *ad libitum* (Chapter 2.4).

3.2.2 IBV challenge strains

A virulent strain of IBV M41 was used as the challenge virus. This virus was propagated and titrated in 10 days old, SPF ECE at 37 °C (Chapter 2.1). The eggs were inoculated via the allantoic cavity as previously described (Bijlenga *et al.* 2004). Virus-rich allantoic fluid was collected and titrated ($10^{5.75}$ CD_{50}/bird) in chicken tracheal organ cultures (TOCs) according to standard procedures (Cook *et al.* 1976) and expressed in 50% median ciliostatic doses (CD_{50}/ml) as previously described (Reed & Muench 1938) (Chapter 2.2). The collected allantoic fluid was confirmed to be free from other viruses by RT-PCR and free of bacterial or fungal contaminants by culture (Chapter 2.6).

3.2.3 Experimental design

Sixty, day-old chicks were divided into two groups (n=30 per group) and distributed as follows: (i) unchallenged (control, n=30) and (ii) challenged at 21 days old (n=30). At 21 days old, each chicken in the challenged group received 0.1 ml of $10^{5.75}$ CD_{50}/bird of IBV via the oculonasal route. Chickens in the unchallenged group were mock challenged using the same route with 0.1 ml of virus-free allantoic fluid to serve as controls. At 1, 2, 3, 4 and 5 days post challenge (dpc), clinical signs were recorded and five chicks from each group were sampled. Oropharyngeal (OP) and cloacal (CL) swabs were collected for virus detection by RT-PCR. Lachrymal fluid was collected to assay for anti-IBV antibodies by monoclonal ELISA, and blood was collected via the brachial vein. Serum was separated from the whole blood, collected and stored at -20 °C. The five birds from each group were then humanely euthanised using sodium pentobarbitone to assess tracheal ciliary health and for tissue sample collection. Harderian gland (HG), choanal cleft, turbinate, pharyngeal tissue and trachea samples were collected and stored in RNA*Later* to preserve the integrity of tissue RNA (Qiagen, Crawley, UK) for the determination of viral load and host gene expression. Samples from the same tissues, except pharyngeal tissue, were also collected for immunohistochemistry (IHC) to demonstrate the replication of the virus. Tracheal portions were also collected in 10% buffered formalin and examined by histopathology. Tracheal ciliary health was assessed using tracheal rings at the point of sampling using a light microscope.

3.2.4 Tracheal ciliary health

Tracheas were collected from each bird during the necropsy and processed for ciliary assessment as previously published (Awad *et al.* 2016c; Cook *et al.* 1999; de Wit *et al.* 2011b), and the mean ciliary score for each bird was used to calculate the ciliary health percentage for each group (Awad *et al.* 2016c; Cook *et al.* 1999) (Chapter 2.7).

3.2.5 Swab samples

Five OP swabs and five CL swabs were taken from each group at all sampling time points. Swabs from the same group were pooled, dipped into 1.5 ml of TOC medium and stored at 70 °C until required (Chapter 2.8.1).

3.2.6 Histopathology

For histopathology examination, the proximal trachea was sampled and fixed in 10% buffered formalin for 24-48 hours before being processed for histological examination and several parameters scored 0-3 as described (Chen *et al.* 1996; Chhabra 2016b) (Chapter 2.11).

3.2.7 Molecular detection of IBV

3.2.7.1 Extraction of RNA (swab)

RNA was extracted from OP and CL swabs using the QIAamp viral RNA mini kit (Qiagen, UK), following the manufacturer's instructions (Chapter 2.12.2).

3.2.7.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

Following extraction, RT-PCR was conducted on swabs to detect a partial IBV S1 sequence (393bp) as previously described (Cavanagh *et al.* 1999; Ganapathy *et al.* 2015) (Chapter 2.14).

3.2.7.3 Extraction of RNA (tissue)

Total RNA was extracted from all collected tissues using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions (Chapter 2.13).

3.2.8 Quantitative reverse transcription polymerase chain reaction (qRT-PCR) for viral RNA quantification

Viral load was quantified from RNA extracted from the HG, choanal cleft, turbinate, pharyngeal tissue, trachea, and swabs. Quantification of viral RNA was carried out by quantitative real-time RT-PCR (qRT-PCR), targeting an IBV 3' untranslated region (UTR) gene-specific primer and probe as previously described (Chhabra *et al.* 2018). Obtained Ct values were converted to log relative equivalent units (REU) of viral RNA by a standard curve generated from using five 10-fold dilutions of RNA extracted from M41 virus-positive allantoic fluid (Londt et al., 2013) (Chapter 2.20).

3.2.9 Host gene expression

Host gene expression analysis was carried out for samples collected at 1-5 dpc. The cDNA created from each extract was subjected to qRT-PCR in triplicate using the LightCycler 480 SYBR Green I Master mix and primers (Chapter 2.21, Table 2.5). Data from qRT-PCR were normalised to 18S ribosomal RNA expression using a relative standard curve method (Chhabra *et al.* 2018; Kuchipudi *et al.* 2012; Okino *et al.* 2013; Rajesh *et al.* 2016), and the data were presented as a fold change difference in gene expression of virus- versus mock-challenged samples.

3.2.10 Serum for humoral antibody detection

Blood samples from the brachial vein were obtained from five birds of each group at 5 dpc and placed in labelled 5 ml bijous without coagulant overnight at room temperature. Serum was stored at -20 °C until further use (Chapter 2.8.2).

3.2.11 Lachrymal fluid for local antibody detection

Lachrymal fluids were collected at 1–5 dpc using sodium chloride and immediately centrifuged at 3000 x g for 5 mins before the supernatant was stored at -20 °C as previously described (Ganapathy *et al.* 2005) (Chapter 2.8.3).

3.2.12 Partial purification of IBV antigen and quantification using the Bradford assay

The IBV M41 was propagated in 10 days old SPF ECE. Partial purification of the antigen was carried out on 25 ml of allantoic fluid by centrifuging the liquid at 1,150 × g, 4 °C for 10 mins. The pellets were washed, re-suspended in phosphate-buffered saline (PBS) and filtered through a 0.22 µm filter. The virus suspensions were overlaid onto 30% sucrose and ultra-centrifuged at 102,400 × g, 4 °C for 4 h. Pellets were stored at -70 °C until use (Ganapathy *et al.* 2005; Keep *et al.* 2015) (Chapter 2.16). The total concentration of purified antigen protein was quantified using the 5x Bradford assay, with 1:10 serial dilutions of bovine serum albumin (BSA), then purified antigen protein concentration was calculated after comparing the optic density (OD) to the BSA standard curve (Chapter 2.17).

3.2.13 Serology

3.2.13.1 Anti-IBV antibody detection by ELISA

A commercial IBV ELISA kit (IDEXX, Westbrook, Maine, USA) was used to determine anti-IBV antibodies for all groups according to the manufacturer's guidelines, with a positive ELISA titre cut-off determined to be 396 (Chapter 2.18.1).

3.2.13.2 Measurement of local antibodies against IBV

Lachrymal fluid for IBV-specific IgA and IgY at 1–5 dpc was assayed using an indirect ELISA (Dhinakar Raj & Jones 1996c; Ganapathy *et al.* 2005; Mockett & Cook 1986). The flat-bottom 96-well microplate (STARLAB®, UK) was pre-coated with partially purified

IBV M41 antigen which had been propagated in 10 days old chicken embryo eggs (Chapter 2.1). The Purified IBV M41 was used as a coating antigen at a concentration of 2.5 µg/ml in a 50 mM sodium carbonate/bicarbonate coating buffer (CBC) with a pH of 9.6. Corrected optical density (COD) values were calculated by deducting the OD values of non-antigen-coated (blank) wells from those of the test wells (Fournier-Caruana *et al.* 2003; Ganapathy *et al.* 2005) (Chapter 2.18.2).

3.2.14 Immunohistochemistry

To detect the IBV antigen, samples of the HG, turbinate, choanal cleft and trachea were collected from five chicks per group, on each day until 5 dpc. All samples were immediately stored in optimal cutting temperature (OCT[43]) and frozen in liquid nitrogen at -190 °C, as previously described (Chhabra *et al.* 2015b). Samples were cut at 5-8 µm thickness and mounted on a microscopic glass slide[43] using a pre-cooled cryostat[44], covered with a glass slide and kept in the dark overnight. After blocking, sections were incubated overnight at 4 °C in the dark with an anti-IBV nucleoprotein monoclonal primary antibody (mouse monoclonal antibody[45]) diluted to 1:20,000 using commercial antibody diluent[46] to detect IBV-positive cells. Using the VECTASTAIN Elite ABC kit[47], the biotinylated anti-mouse IgG was employed to detect the primary antibody, and then a 4% 3, 3-diaminobenzidine solution containing 2% H_2O_2 and 2% buffer stock solution was used to develop the colour reaction (Abdel-Moneim *et al.*

[43] Solmedia laboratory, Shrewsbury, UK
[44] LEICA CM 1900, Germany
[45] Prionics, Lelystad, the Netherland
[46] Dako EnVension Flex, Glostrup, Denmark
[47] PK-6100, Vector Laboratories, Burlingame, USA

2009). Sections treated in the absence of the primary antibody were used as negative controls.

The stained cells were counted in three selected microscopic fields at 400x magnification, and the intensity of staining in each tissue was scored as none (0), mild (1), moderate (2) or severe (3). The mean score of the infected cell count data was expressed as an antigen score (Oladele *et al.* 2009) (Chapter 2.15).

3.2.15 Statistical analysis

Data were confirmed to be normally distributed and analysed using either Student's t-test (two groups) or a one-way analysis of variance (ANOVA), followed by the post hoc LSD multiple comparison test using GraphPad™ Prism version 6.00. Differences between groups were considered significant at $P<0.05$ (Chapter 2.22).

3.3 Results

3.3.1 Clinical signs

No clinical signs were seen in the unchallenged control group during the experimental period. The M41-challenged chickens exhibited mild clinical signs at 3–5 dpc consisting of râles, coughing, sneezing, nasal discharge and open mouth breathing (Figure 3.1, A and B).

3.3.2 Gross lesions

At necropsy, no gross lesions were found in the control group. Gross lesions in the challenged chickens consisted of congestion and excess of mucous in the trachea at 3 and 5 dpc (Figure 3.1, C and D).

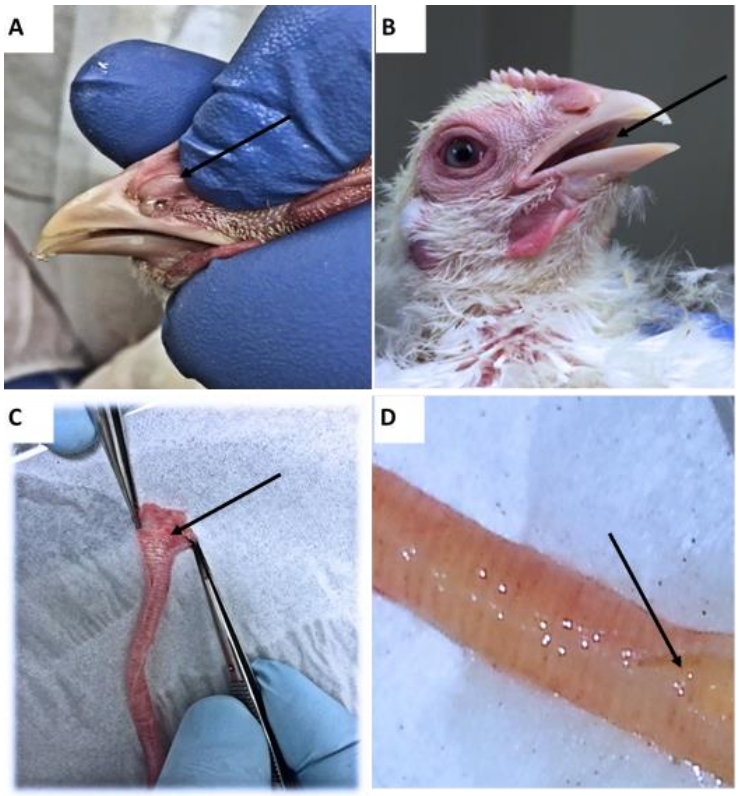

Figure 3.1. Development of the clinical signs and gross lesion after IBV M41 inoculation to 21-day-old commercial broiler chicks. Arrows showing, (A) Nasal discharge and exudates. (B) Open mouth breathing. (C) Tracheal congestion after 3 days of infection. (D) Excessive mucous in the trachea at 5 dpc.

3.3.3 Tracheal ciliary health

The challenged group demonstrated that average ciliary activity declined from 90% at 1 dpc to 1.5% by 5 dpc, whereas the unchallenged group remained above 99% for all sampling days (Figure 3.2).

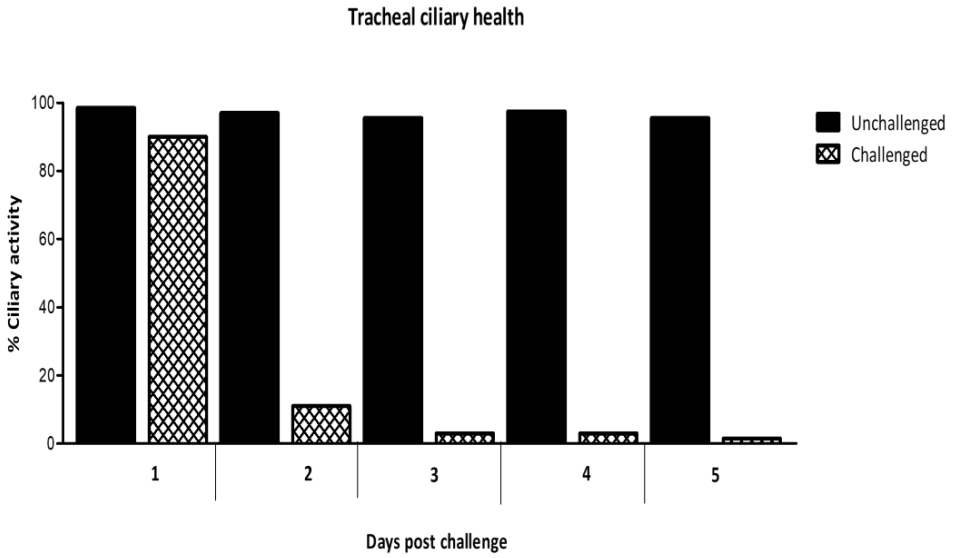

Figure 3.2. Tracheal ciliary activity in the IBV M41 challenge group of chickens over the period from 1–5 dpc.

3.3.4 Histological changes

The chickens in the unchallenged control group exhibited unaltered tracheal epithelium, with healthy cilia and goblet cells (Figure 3.3 A and B). In the challenged group, tracheal lesions mainly consisted of ciliary loss (deciliation), decreased mucous cells, degeneration of epithelium, and heterophilic and lymphoid infiltrations (Figure 3.3 C-F). The highest tracheal histopathology scores were seen at 3 and 4 dpc with no significant difference in changes between the challenged group at any time point (Figure 3.4). Tracheal lesions started early, at 1 dpc, and remained until 5 dpc. Lesion severity peaked by 3 dpc with a mean score of 12.6±0.67 (Table 3.1).

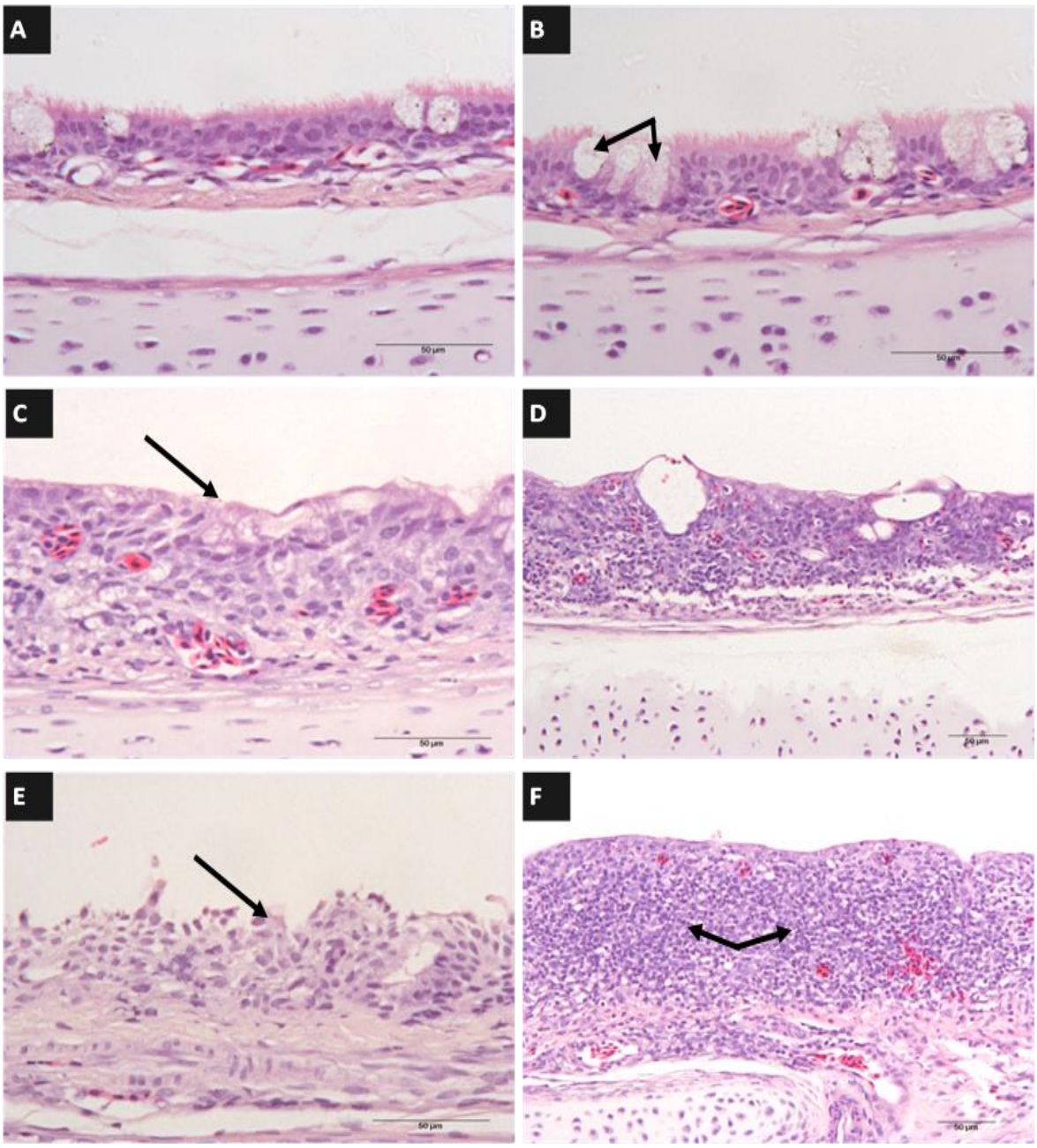

Figure 3.3. Arrows showing tracheal histopathological findings, using haematoxylin and eosin (H&E) stain. (A and B): Unchallenged control group (score: 0) exhibiting intact cilia (A) and goblet cells (B). (C–F): Challenged group (score: 3) exhibiting complete epithelial deciliation (C), decrease in mucous cells (D), epithelial degeneration (E) (magnification at 400x), and severe infiltration by plasma cells and lymphocytes (F; magnification 100x).

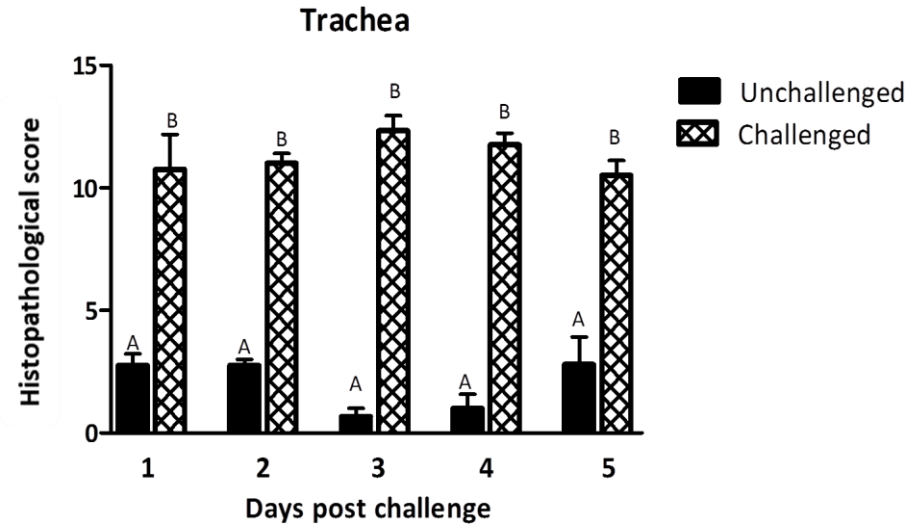

Figure 3.4. Histopathological scores of lesions in the trachea of unchallenged and challenged groups. Birds were challenged with the IBV strain M41 at a dose of $10^{5.75}$ CD_{50}/bird and were processed for histopathological examination at 1–5 dpc. Data are presented as the average mean of total scores for each parameter with standard error bars. One trachea section was scored/bird. Significant differences between the groups indicated with different letters.

Table 3.1. Histopathology of the trachea in unvaccinated broiler chicks challenged with the IBV M41 strain. The scores for each histopathological criterion in each bird were added together and the mean score for the birds at each time point was calculated. The mean histopathological lesion score is expressed as ± SEM. (1, 2 and 4 dpc n =3; 3 and 5 dpc n=5).

Days post challenge	Bird No.	Epithelial deciliation	Epithelial degeneration	Decrease in mucous cells	Heterophil infiltration	Epithelial hyperplasia	Lymphoid infiltration	Total score	Mean score ± SEM
1	**1**	2	2	3	2	0	2	11	**10.6±3.5**
	2	3	3	3	3	0	2	14	
	3	1	1	2	1	1	1	7	
2	**1**	2	2	3	1	1	2	11	**11±0.57**
	2	3	1	2	1	1	2	10	
	3	3	2	2	2	1	2	12	
3	**1**	2	2	2	1	2	2	11	**12.6±0.67**
	2	2	1	2	2	2	2	11	
	3	3	2	3	1	2	3	14	
	4	3	2	3	2	1	2	13	
	5	3	2	3	2	2	2	14	
4	**1**	3	2	2	1	1	2	11	**12±0.57**
	2	3	2	2	1	2	2	12	
	3	3	2	2	2	2	2	13	
5	**1**	2	2	1	0	2	2	9	**10.8 ± 0.6**
	2	2	3	2	1	1	2	11	
	3	3	3	2	1	2	2	13	
	4	2	2	2	0	2	2	10	
	5	2	2	3	0	2	2	11	

3.3.5 Detection of IBV from OP and CL swabs by RT-PCR

Throughout the study period, no virus was detected in the control group. The viral genome was detected by RT-PCR in the pooled OP and CL swabs from the M41-challenged group for all time points (1–5 dpc).

3.3.6 Systemic humoral immune responses by ELISA

High levels of IBV maternal-derived antibody (MDA) were detected in the day-old control birds, with the mean antibody titre being 1376±386. At 5 dpc (26 days-old), both the unchallenged and challenged groups remained negative with titres of 21 and 90.6 respectively (Figure 3.5).

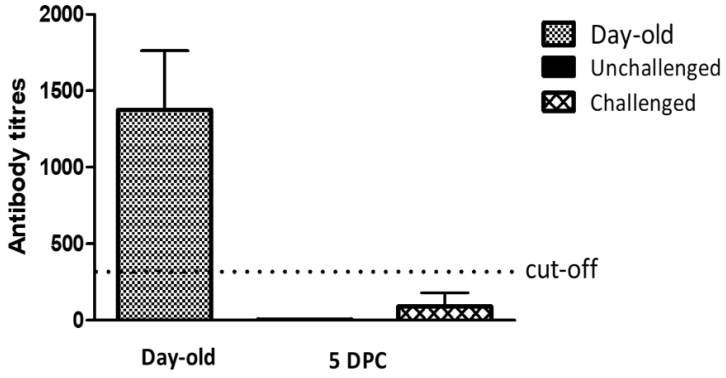

Figure 3.5. Mean anti-IBV ELISA antibody titres in the M41-challenged and unchallenged chicks at 5 dpc. The ELISA cut-off titre is 396.

3.3.7 Mucosal immune responses (IgA and IgY by indirect ELISA)

The level of specific anti-IBV IgA gradually increased in lachrymal fluid from 1–5 dpc after M41 infection. Compared to the unchallenged control group, a significantly higher titre was found in the challenged group at 4 and 5 dpc (0.095±0.018 and 0.117±0.021, respectively). Similar significant increases and comparisons were also found in IgY levels at same time-points (0.053±0.020 and 0.070±0.019, respectively) (Figure 3.6).

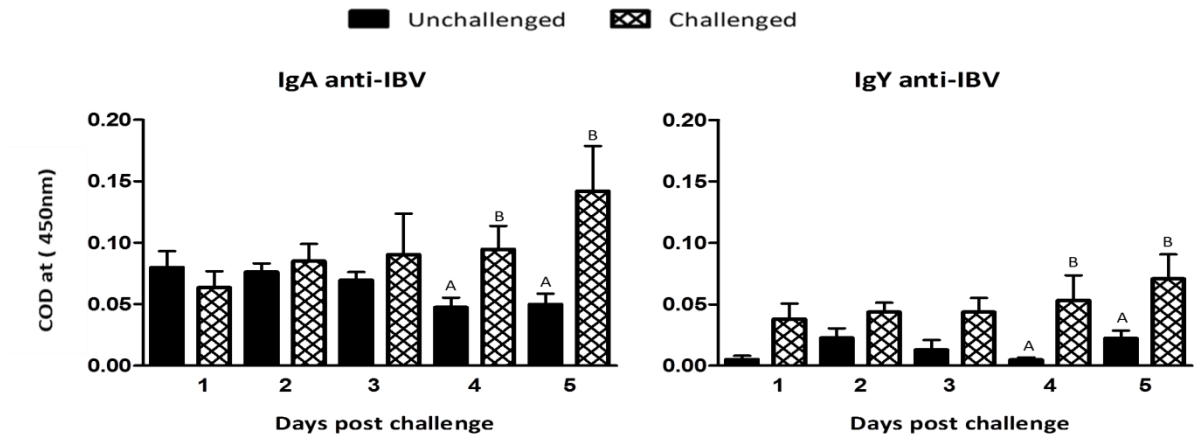

Figure 3.6. Measurement of IBV-specific IgA and IgY titres using an indirect ELISA from lachrymal fluid collected at 1–5 dpc. Data are expressed as the mean of corrected optical density (COD). Significant differences within the same sampling day are indicated with different letters (n=5).

3.3.8 Viral load quantification from swabs

Viral RNA loads in all swabs in the unchallenged control group were below the detection limit on all days post challenge, whereas all swabs samples collected from challenged birds were positive on all sampling days (Figure 3.7). The viral load of the oropharyngeal swabs peaked at 1 dpc (2.36 log REU). Viral load in the cloacal swabs peaked late, at 5 dpc (2.54 log REU) (Figure 3.7).

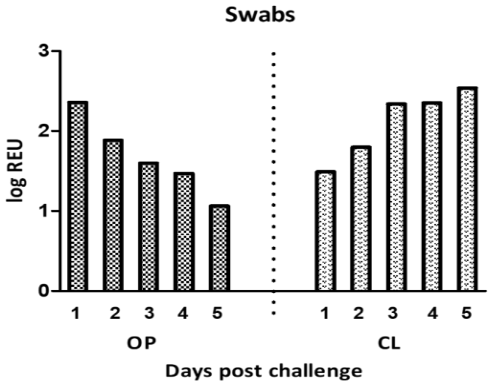

Figure 3.7. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA in challenged groups. IBV loads are displayed at 1, 2, 3, 4 and 5 dpc in OP and CL swabs.

3.3.9 Viral load in tissues by qRT-PCR

All tissue samples (the HG, turbinate, choanal cleft, tracheal and pharyngeal tissues) collected from the control (unchallenged) group were below the detection limit (negative), whereas all tissue samples collected from the challenged birds were positive on all sampling days (Figure 3.8 A–E). The viral load in the turbinate, choanal cleft and pharyngeal tissue peaked at 3 dpc (3.64, 3.38 and 3.45 log REU, respectively), with a significant increase at 3–5 dpc in the turbinate and at 2–4 dpc in the choanal cleft and pharyngeal tissue. The viral load peaked earlier in the HG and tracheal tissues at 2 dpc (3.57 log REU and 3.92 log REU, respectively) with a significant increase at 2-4 dpc in the HG. The tracheal viral load peaked at 2 dpc (3.91 log REU) with significant increases between 1, 4 and 5 dpc. There was a significantly lower viral load in the pharyngeal tissue at 1 dpc in comparison to other tissues. In the trachea and pharyngeal tissues, a significantly lower viral load was found at 4 dpc compared to other tissues, and at 5 dpc compared to the turbinate and choanal cleft (Figure 3.8 F).

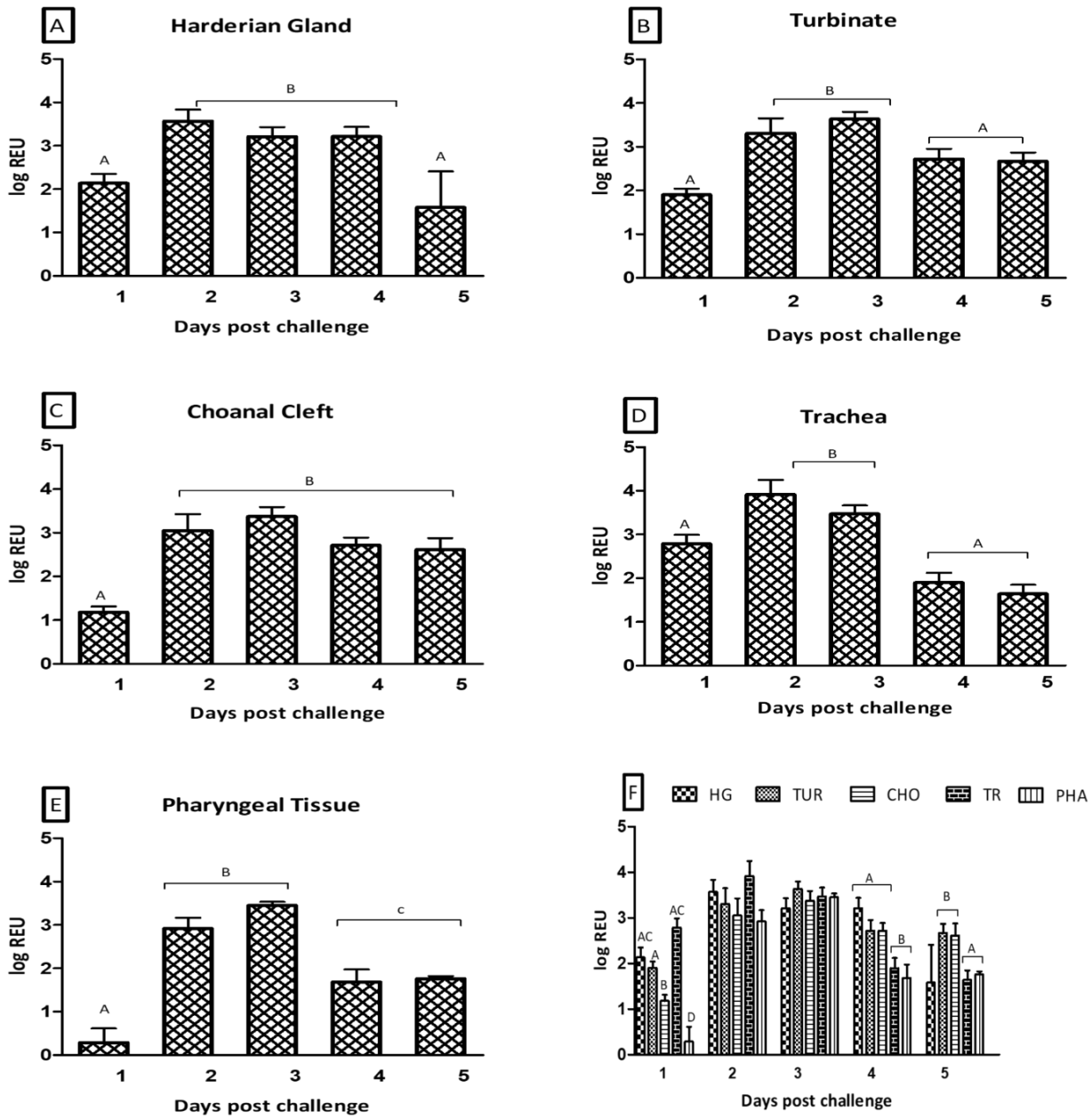

Figure 3.8. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA in (A) Harderian gland, (B) the turbinate, (C) the choanal cleft, (D) the trachea and (E) the pharyngeal tissue; (F) presents a comparison between the tissues on the same day. Significant differences between the groups are indicated with different letters ($P<0.05$).

3.3.10 Quantification of IBV replication by immunohistochemistry (IHC)

Images from the unchallenged group confirmed the absence of background and non-specific staining (Figure 3.9 A1–D1). The presence of IBV M41 antigen was demonstrated in epithelial tissue of the turbinate, choanal cleft, HG and trachea (Figure 3.9 A2–D2).

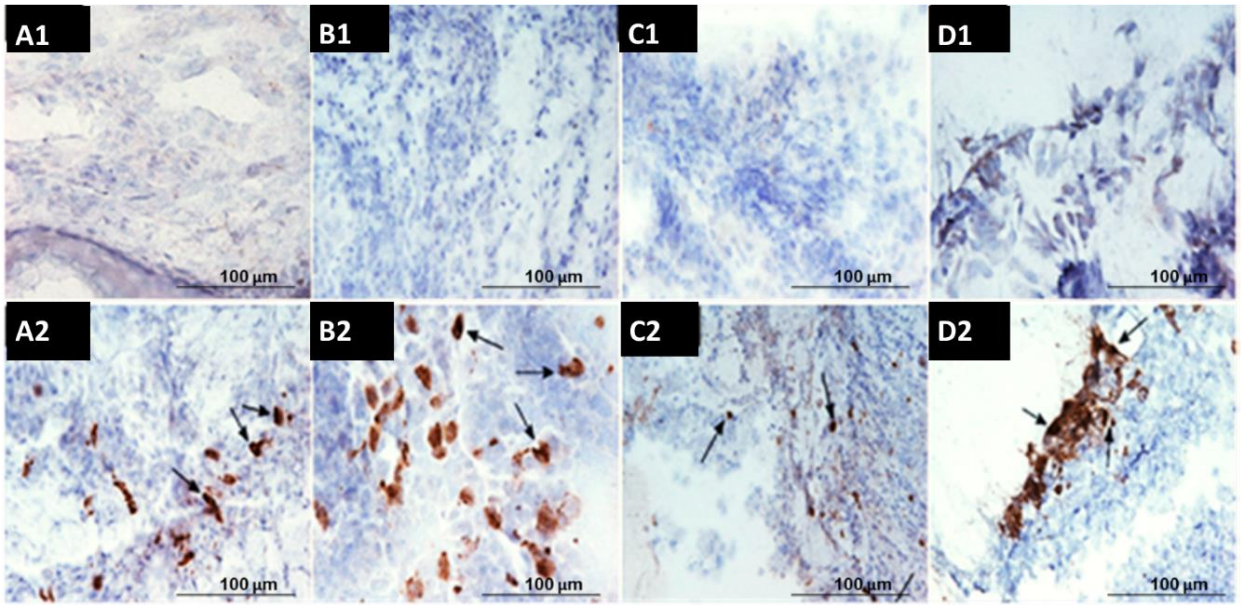

Figure 3.9. M41 virus within tissues at 5 dpc, using diaminobenzidine (DAB), and haematoxylin stain. A1–D1: Tissues from unchallenged birds; turbinate, choanal cleft, Harderian gland and trachea, respectively. A2–D2: Challenged birds showing the presence of IBV in the turbinate, choanal cleft, Harderian gland and trachea, respectively. Arrows indicate some of the infected cells in which staining indicates the presence of IBV M41. Mouse monoclonal antibody against IBV nucleoprotein was used to detect IBV antigen.

Antigen scores of the HG, turbinate, choanal cleft and trachea tissues peaked at 3 dpc (Figure 3.10). There was a significant increase at 3 dpc compared with all other sampling days in the HG, and a significant increase between 1 and 3 dpc in the turbinate. In the choanal cleft, there was a significant decrease in the antigen score after 3 dpc, whereas

the trachea presented a significant increase at the same point in time. The only significant differences between tissue types were seen at 5 dpc, when the trachea had a significant decrease in the antigen score compared with the HG, turbinate and choanal cleft.

All unchallenged control tissues contained less than 5 stained cells per x400 objective lens of the light microscope, and were scored as 0.

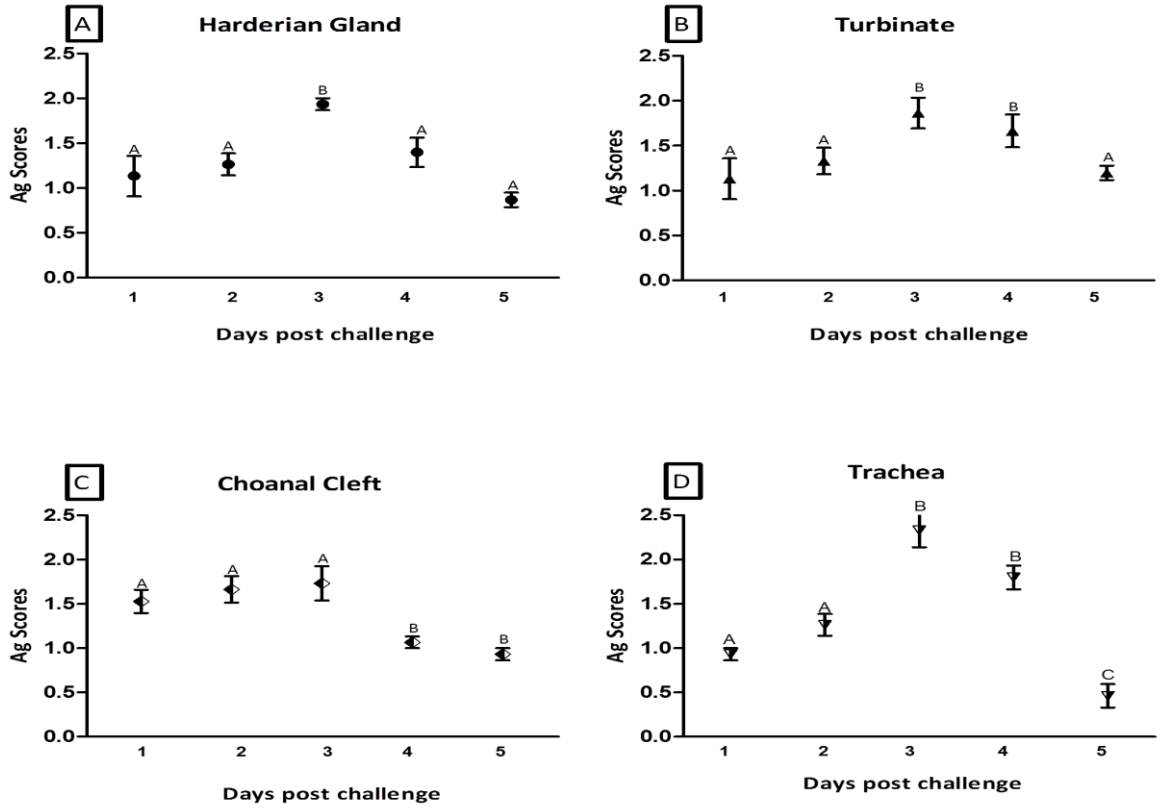

Figure 3.10. Immunohistochemistry (IHC) staining scores of the IBV M41 virus in the Harderian gland, turbinate, choanal cleft and trachea tissues samples. The number of challenged cells for the birds (n=5/group) were analysed at 1, 2, 3, 4 and 5 dpc. Data are represented as the mean, with error bars indicating the standard error. Significant differences between the groups are indicated with different letters ($P<0.05$).

3.3.11 Expression of host TLR3 and MDA5 mRNA

Harderian gland (HG)

There was significant up-regulation in the mRNA expression of TLR3 at 2 dpc compared with the control group, with no other significant changes on the other sampling days (Figure 3.11 A). There was a significant up-regulation in the mRNA expression of MDA5 at 2–5 dpc compared with the control group (Figure 3.12 A).

Choanal cleft

The mRNA expression of TLR3 was significantly up-regulated at 1 dpc compared with the control group, with no other significant changes observed in the other sampling days (Figure 3.11 C). The mRNA expression of the MDA5 gene presented significant up-regulation at 1 and 2 dpc compared with the control group (Figure 3.12 C).

Turbinate

In the mRNA expression of TLR3, there was significant up-regulation at 1 dpc compared with the control group (Figure 3.11 B). The expression of MDA5 in the challenged group had significant up-regulation only at 1 and 2 dpc compared with the control group (Figure 3.12 B). No further significant changes were noticed for the other sampling days beyond 2 dpc.

Trachea

The up-regulation of TLR3 mRNA expression was significant at 1 and 2 dpc compared with the control group (Figure 3.11 D). The expression of MDA5 in the challenged group was only significantly up-regulated at 1 dpc (Figure 3.12 D). No significant changes were observed in the other sampling days for both genes when compared with the control group.

Pharyngeal tissue

The mRNA expression of TLR3 was significantly up-regulated at 1 and 5 dpc compared with the control group (Figure 3.11 E). The mRNA expression of the MDA5 gene exhibited significant up-regulation at 1 and 2 dpc compared with the control group, with no other significant changes noticed on the other sampling days (Figure 3.12 E).

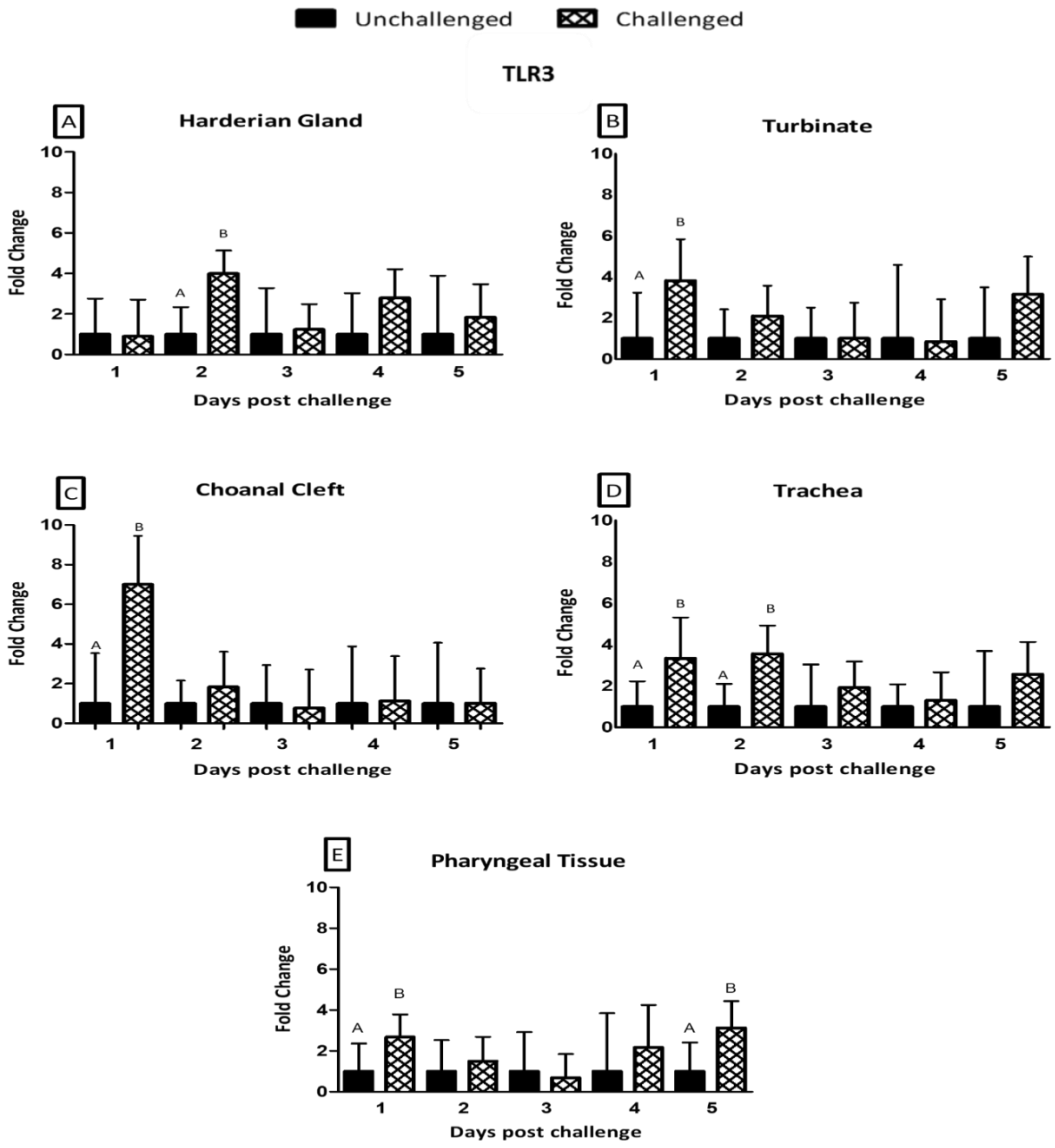

Figure 3.11. Relative mRNA expression of TLR3 (A–E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue sample of chickens. Data represent the mean (n=5), with error bars indicating standard errors, and are expressed as fold changes relative to the unchallenged control group. Significant differences between the groups are indicated with different letters ($P<0.05$).

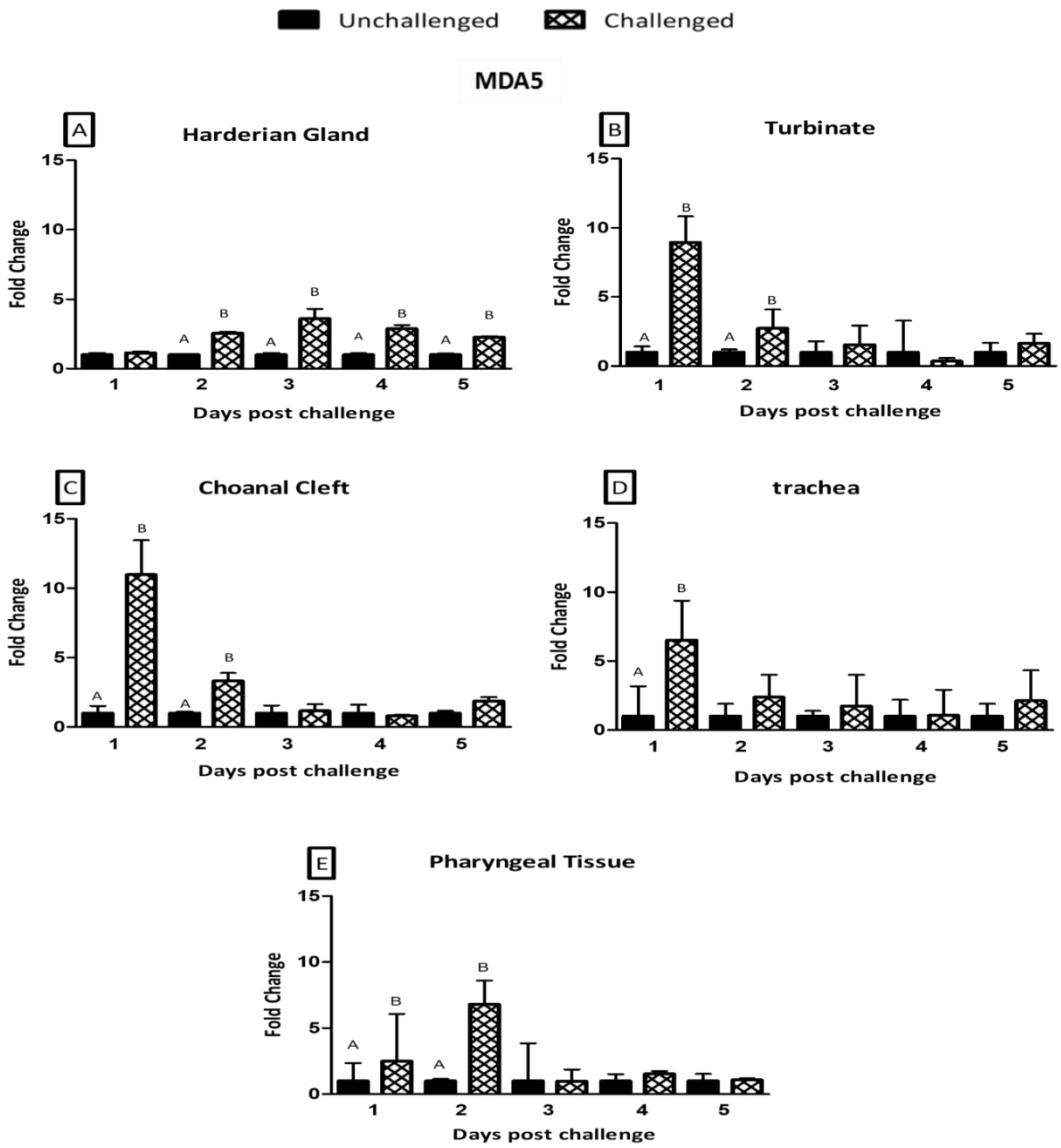

Figure 3.12. Relative mRNA expression of MDA5 (A–E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue sample of chickens. Data represent the mean (n=5), with error bars indicating standard errors, and are expressed as fold changes relative to the unchallenged control group. Significant differences between the groups are indicated with different letters ($P<0.05$).

3.3.12 Expression of host IFN-α and IFN-β mRNA

Harderian gland

Infection with IBV M41 resulted in significantly greater IFN-α expression in the HG at 3 dpc compared with the control group. No significant changes in the level of IFN-α expression were observed at the other time points (Figure 3.13 A). There was down-regulation in the mRNA expression of IFN-β at 1 dpc, then significantly higher expression was seen at 3–5 dpc compared with the control group (Figure 3.14 A).

Choanal cleft

The expression level of IFN-α mRNA was only significantly up-regulated at 1 dpc compared with the control group. No significant changes in the level of IFN-α expression were observed beyond 1 dpc (Figure 3.13 C). Expression of IFN-β mRNA was significantly down-regulated at 1 dpc, followed by significant up-regulation at 2 dpc when compared with control group. No significant differences were seen beyond 2 dpc (Figure 3.14 C).

Turbinate

Expression of IFN-α was significantly up-regulated at 1 dpc. Down expression of this gene was apparent at 5 dpc, with no significant changes at other sampling points compared with the control group (Figure 3.13 B). The level of IFN-β mRNA expression was initially down-regulated at 1 dpc, followed by a significant up-regulation at 2 dpc compared with the control group (Figure 3.14 B).

Trachea

There was significant up-regulation in the mRNA expression of IFN-α at 1, 2 and 3 dpc followed by a reduction to base levels at 4 and 5 dpc compared with the control group (Figure 3.13 D). Expression of IFN-β mRNA was significantly down-regulated at 1 dpc

compared with control group. The significant expression was significantly up-regulated at 2 and 3 dpc after a comparison was made with the control group (Figure 3.14 D).

Pharyngeal tissue

There was a significant up-regulation in the mRNA expression of IFN-α at 2 dpc, followed by significant down-regulation at 3 dpc compared with the control group (Figure 3.13 E). Expression of IFN-β mRNA was significantly down-regulated at 1 dpc, followed by significant up-regulation at 2 dpc compared with the control group. No significant differences were observed beyond 2 dpc (Figure 3.14 E).

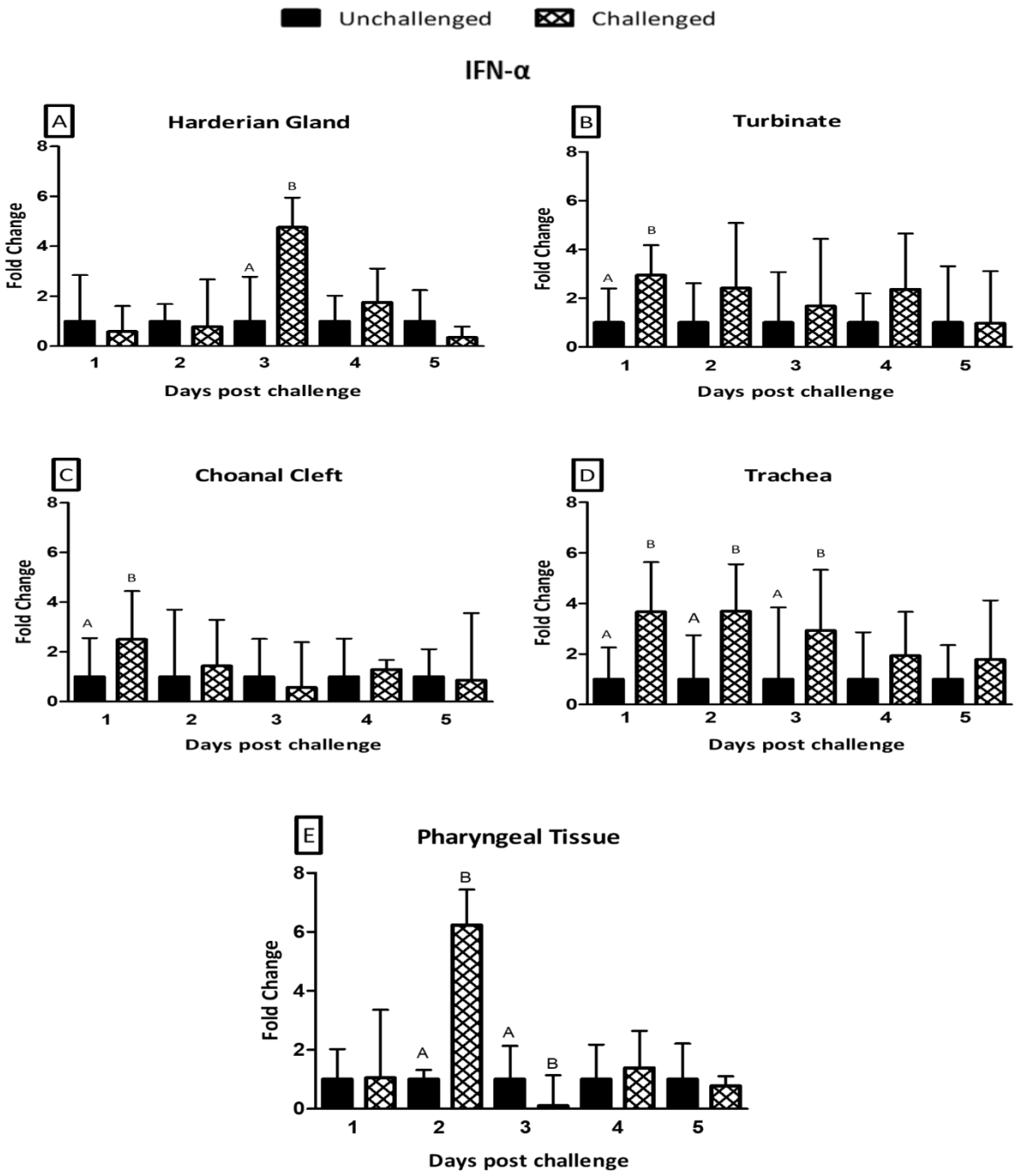

Figure 3.13. Relative mRNA expression of IFN-α (A–E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue samples of chickens. Data represent the mean (n=5), with error bars indicating standard error, and are expressed as fold changes relative to the unchallenged control group. Significant differences between the groups are indicated with different letters ($P<0.05$).

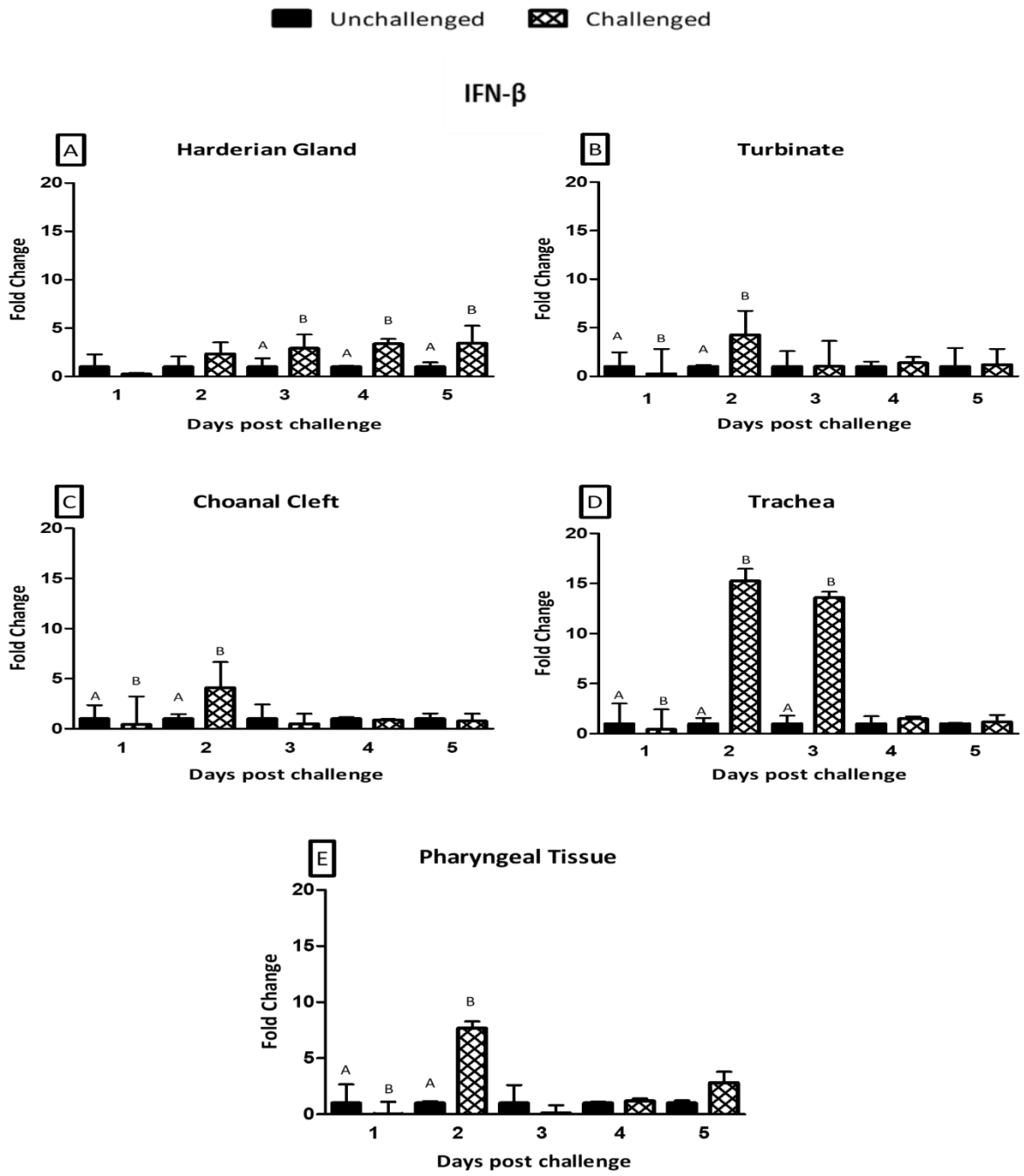

Figure 3.14. Relative mRNA expression of IFN-β (A–E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue samples of chickens. Data represent the mean (n=5), with error bars indicating standard error, and are expressed as fold changes relative to the unchallenged control group. Significant differences between the groups are indicated with different letters ($P<0.05$).

3.3.13 Pro-inflammatory cytokine (IL-6) mRNA expression

Harderian gland

The results indicate that the level of IL-6 mRNA expression was significantly down-regulated at 1 dpc, with mRNA expression significantly expressed at 3 dpc compared with the control group (Figure 3.15 A).

Choanal cleft

Expression of IL-6 mRNA was significantly higher in the choanal cleft at 1, 2 and 5 dpc in the challenged group compared with the control group (Figure 3.15 C).

Turbinate

Expression of IL-6 mRNA was significantly higher at 1, 2 and 5 dpc in the challenged birds compared with the control group (Figure 3.15 B).

Trachea

There was a significant up-regulation in the mRNA expression of IL-6 at 1 and 2 dpc in the challenged group with no other significant changes observed on the other sampling days compared with the control group (Figure 3.15 D).

Pharyngeal tissue

Expression of IL-6 mRNA was significantly higher at 2 and 5 dpc in challenged birds compared with the control group (Figure 3.15 E).

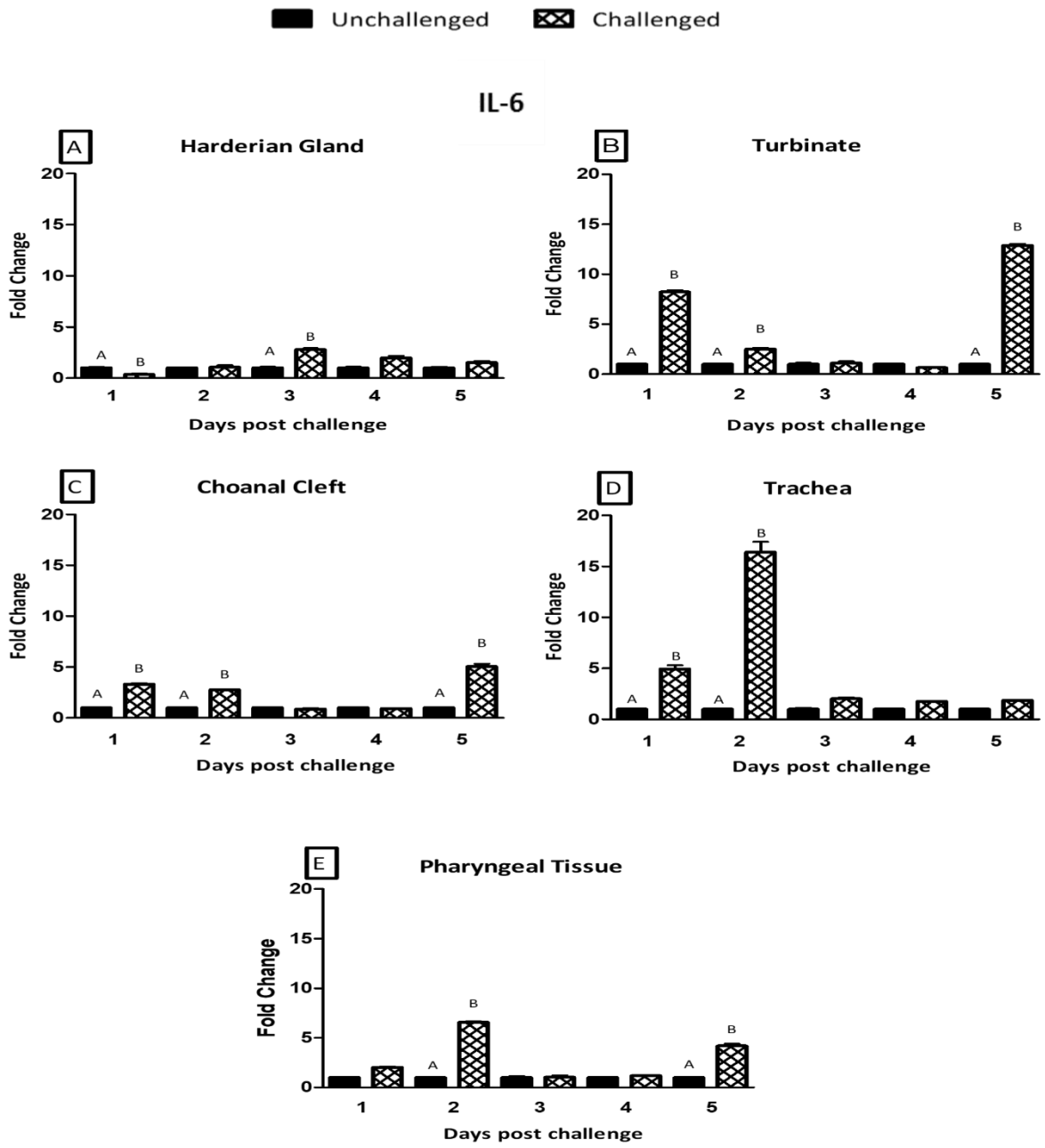

Figure 3.15. Relative mRNA expression of IL-6 (A–E) in the Harderian gland, turbinate, choanal cleft, pharyngeal tissue and trachea samples of chickens. Significant differences between the groups are indicated with different letters (P<0.05). Data represent the mean (n=5), with error bars indicating standard error, and are expressed as fold changes relative to the unchallenged control group.

3.4 Discussion

This is the first study to investigate the effects of classical virulent M41 on the expression of innate antiviral mediators in head-associated lymphoid (HG and choanal cleft) in comparison to respiratory (turbinate and tracheal) tissues. While a number of studies have demonstrated host-virus interactions using virulent IBV strains (Chhabra *et al.* 2018; He *et al.* 2016; Okino *et al.* 2014), there is limited work to date that has investigated the broiler chicken's innate immune response in tissues other than the trachea. Therefore, this study was carried out to mimic the natural air passages, where vaccine or virulent viruses including IBV M41, passes through the turbinate, HALT then reaching the trachea. Host-virus interactions in naïve and M41-infected chicks were examined.

There was a gradual reduction of cilia activity in the challenged birds from 2–5 dpc. Moreover, severe histological changes in the trachea were also observed between 2–5 dpc, manifesting as deciliation and degeneration of epithelium, decreased numbers of mucous cells, and heterophil and lymphoid infiltration. Histopathological scores in the trachea during this time period were high and associated with viral load and reduction in ciliary activity. This suggests a role of virus load on the development of tracheal lesions. These findings support the results of previous work involving the IBV M41 strain (Chhabra *et al.* 2018; Okino *et al.* 2014).

Systemic anti-IBV antibodies in the challenged chicks were negative at 5 dpc. Virus-neutralising antibodies are likely directed against spike proteins and not against nucleocapsid and membrane proteins (Hasony & Macnaughton 1981). In accordance with the present results, previous studies have demonstrated that antibodies can be detected for the first time at seven days of vaccination or infection (Hawkes *et al.* 1983;

Mockett & Darbyshire 1981). A possible explanation for this is that anti-spike antibodies are present during early infection, but are not directed at the antigenic determinants involved in virus neutralisation (Hawkes *et al.* 1983).

The presence and importance of IBV-specific IgA antibodies to mucosal immunity in the upper respiratory tract and lachrymal fluid has been previously described (Gelb *et al.* 1998; Toro & Fernandez 1994). The detection of IgA and IgY after infection with IBV peaks at 7 days post-infection (Raj & Jones 1996b). By examining the early production of lachrymal IgA following M41-infection, this study has, for the first time, demonstrated significantly higher antibody levels at 4–5 dpc in the challenged birds compared to the control group. Suggesting that the induction of IBV-specific IgA and IgY in challenged birds could be used as a biomarker of exposure to IBVs. This study indicates that IBV-specific IgA detection could be refined in the near future and may act as a useful tool in the early detection of IBV field outbreaks, although such an application would require further investigation. It is likely that the role of these antibody isotypes is essential for mucosal immunity against IBV infection of the upper respiratory tract (Chhabra *et al.* 2015b; Da Silva *et al.* 2017; Gelb *et al.* 1998; Okino *et al.* 2013; Toro & Fernandez 1994).

Though the tracheal epithelium is important tissue for replication, the virus first progresses through other respiratory tissues such as the turbinate, conjunctiva, infraorbital sinuses, HG, choanal cleft and pharyngeal tissues. This study demonstrated high virus load in such tissues, and the higher viral load during 1–3 dpc indicates rapid dissemination to the investigated tissues during the viraemic stage. Previous data has also suggested the maximum virus yield was reached at between 48–72 hours in the

nasal turbinate and between 24–48 hours in trachea (Darbyshire *et al.* 1978). In addition, using in-situ hybridization, Dolz *et al.* (2012) demonstrated the presence of viral RNA in the nasal turbinate with extensive replication at 1 dpi prior to the trachea. Due to the route of infection, these tissues are likely the most susceptible and respond more rapidly to IBV.

As virus presence alone may not reflect localised replication, for this reason, IHC was employed to further demonstrate such replication in tissues. Similar to previous work, results of this study demonstrated the greatest IHC detection at 2–3 dpc and a significant reduction at 5 dpc, which was associated with peak viral load by qRT-PCR (Abdel-Moneim *et al.* 2009). Data suggests that upper respiratory IBV M41 infection in susceptible chickens is acute, due to the short period of the antigen presence. This may be strain-dependant, however, as according to Benyeda et al. (2009), while M41 viral titres in the trachea dropped to zero by 7 dpc, the virus titres of Greek (D591/2), Chinese (D532/9), Slovakian (D722) and French (D535/4) strains only became negative by 21 or 28 dpc. In accordance with the present results, previous studies have demonstrated that positive antigen levels in the turbinate have been observed between 1–3 dpc after vaccination with IBV H120 (Darbyshire *et al.* 1976).

Past publications has highlighted the benefit of analysing TLR3, MDA5, IFN-α, IFN-β and IL-6 in better understanding the immunopathogenesis of vaccine or virulent IBVs (Chhabra *et al.* 2018; Kameka *et al.* 2014; Okino *et al.* 2017; Wang *et al.* 2006). While previous work has only focused on immune responses in the trachea and kidney (Chhabra *et al.* 2018; He *et al.* 2016; Kameka *et al.* 2014; Okino *et al.* 2017; Okino *et al.* 2014; Wang *et al.* 2006), this is the first study which has attempted to examine the daily

kinetics of early innate immune responses of lymphoid (HG and choanal cleft) and respiratory (turbinate and tracheal) tissue in M41-challenged chickens. Findings showed a clear pattern of mRNA up-regulation of all examined genes was found in the choanal cleft, pharyngeal tissue, turbinate and trachea, except at 1 dpc, where IFN-β was down-regulated in all tissues. Tracheal expression of mRNA was similar to that of previous work (Chhabra *et al.* 2018; He *et al.* 2016; Okino *et al.* 2017) but contrasted the findings of Wang et al. (2006), who found that TLR3 expression increased at 3 dpc following an M41 challenge. Suggesting a role of chicken innate immune responses at the early phase of IBV infection.

Type I IFN responses are an essential defence mechanism against viruses. In the challenged group, both interferons investigated were significantly up-regulated during the early sampling days (1–2 dpc) in the choanal cleft, pharyngeal tissues and turbinate, whereas a delayed response was witnessed in the HG (3 dpc). Similar findings were reported by Okino *et al.* (2017) after a challenge with two Brazilian strains, and Chhabra et al. (2018) following infection with IS/885/00-like, QX-like and M41 IBV strains. The end product of the TLR3 and MDA5 pathways, IFN-β, has been associated with protection against IBV infection, tissue tropism, histopathology and antiviral states (Chhabra *et al.* 2018; Okino *et al.* 2017).

In contrast to other genes, IL-6 mRNA expression in tracheal tissue demonstrated up-regulation at all sampling points, whereas the turbinate only had significant up-regulation 1–2 dpc, followed by another increase at 5 dpc. The reason for the second peak is not known, although similar findings have been reported for trachea and kidney tissues in different chicken lines following infection with IS/885/00-like, QX-like and

M41 IBV strains, or following variant IBV/Brazil/PR05 infection (Asif *et al.* 2007; Chhabra *et al.* 2018; Fernando *et al.* 2015). Recently, IL-6 expression has been associated to tissue damage, increased viral load and increased tissue tropism (Chhabra *et al.* 2018), suggesting that the increased IL-6 production in the current study may have contributed to cilia degradation in the trachea.

To our knowledge, this is the first study which has examined the association between early host innate immune responses in head-associated lymphoid and respiratory tissues in association with cilia viability in naïve chickens challenged with M41. Based on IBV qRT-PCR and IHC, this study demonstrated that other than the trachea, IBV actively replicates in head-associated lymphoid (HG and choanal cleft) and turbinate tissues, which appears to contribute to the infection and immunobiology. However, such effects were dependent on the tissue type, with significant changes in TLR3, MDA5, IFN-α and IL-6 mRNA expression in the turbinate and trachea being most notable. This study also demonstrated the use of IBV-specific IgA and IgY in lachrymal fluid as infection markers. Early up-regulation of these host gene signatures and inflammatory and pro-inflammatory cytokines (including TLR3, MDA5, IFNs and IL-6) in HALT and respiratory tissues, and the lachrymal IgA and IgY levels, could be used as a protection indicator in an IBV vaccination-challenge model. It appears that the cilia-protection of the trachea in IBV-vaccinated-challenged birds could be due to the reduction of challenge IBVs reaching the trachea. Basically, the challenge viruses inhibited by innate, mucosal and cellular immunity in the head-associated lymphoid and respiratory tissues. This was investigated in the Chapter 4.

Chapter4: Immune responses and protection in head-associated lymphoid and respiratory tissues in response to IBV M41 challenge in vaccinated commercial broiler chicks

Abstract

The European Pharmacopeia protocol on infectious bronchitis (IB) vaccine efficacy testing currently over relies on the cilia-stopping test. This test is dependent on subjective reading of tracheal ciliary movement of vaccinated and unvaccinated chickens with or without virulent IBV challenge. While the trachea is an important site of IBV replication, it has been observed that head-associated lymphoid and respiratory tissues are likely to play a greater role in protection against virulent infectious bronchitis virus (IBV). In an attempt to propose new quantitative parameters for assessment of protection in IBV vaccine efficacy studies, the current study aimed to examine early host immune responses using serological and immuno- molecular methods. Day-old broiler chicks were vaccinated via the oculonasal route with 0.1 ml of live IBV vaccines (Mass and 793B), and at 21 dpv chicks were challenged with 0.1 ml of virulent IBV M41 ($10^{5.75}$ CD_{50}/bird). Serum and lachrymal fluid were collected for anti-IBV IgA and IgY using ELISA. Tissue samples (HG, choanal cleft, turbinate, pharyngeal tissue and trachea) were taken and examined for viral load and host gene responses. The vaccinated- challenged group showed significant increases in mucosal immunity as reflected by lachrymal IBV-specific IgA at 3-5 dpc. Only the trachea of vaccinated-challenged birds had a significantly higher viral load at 1-4 dpc. For vaccinated groups, challenged birds had a significantly higher TLR3, MDA5, IFN-α and IL-6 expression in the turbinate, choanal cleft and trachea between 1- 2 dpc. While further investigation is needed, findings from this study have for the first time, provided a scientific foundation for the inclusion of quantitative early immune parameters for protection measurement in IBV vaccination-challenge studies.

4.1 Introduction

Infectious bronchitis virus (IBV) remains one of the main causes of economic losses in the poultry industry, affecting not only the production of meat (Cavanagh 2007), but also chicken health and welfare (DEFRA 2002; Lay *et al.* 2011). In the UK, it was estimated that infectious bronchitis (IB) has caused disease in 22.5 million chickens at a total cost of £23.6 million (DEFRA 2005).

Traditionally, the trachea has been highlighted as the primary tissue for IBV replication (McMartin 1993; Raj & Jones 1997). As a result, in 1976, the tracheal cilia-stopping assay was introduced to assess vaccine-efficacy (Cook *et al.* 1976). In the UK, poultry vaccines are required to meet both Veterinary Medicine Directorate (VMD) and European Medicines Agency (EMA) standards. In line with this, the European Pharmacopoeia (PhEur) commission confirmed that the cilia-stopping test is an indicator of protection against IBV, and vaccinated chickens yielding 50% or more tracheal explants with ciliary activity are considered protected against a virulent challenge (Cook *et al.* 1999; Council of Europe 2007; de Wit *et al.* 2013). Limitations of the test are partially due to subjective reading and also relying solely on the trachea as a protection indicator. Reports describe respiratory clinical signs of sneezing, snicks, stuffiness and head-shaking present in vaccinated-challenged birds with or without ciliostasis (Awad *et al.* 2015; Chhabra *et al.* 2015b; Yang *et al.* 2018). Thus, it would be worthwhile to investigate the role of other tissues, in particular lymphoid and respiratory tissues in the head, as IBV is mostly spread via this route.

Despite this apparent importance of the head-associated lymphoid and respiratory tissues, no attempts have been made to understand the kinetics of viral replication, or cellular and mucosal immune responses in the HALT and respiratory tissues of

vaccinated-unchallenged or vaccinated-challenged birds. Studies investigating protection (Awad *et al.* 2015; Awad *et al.* 2016c; Chhabra *et al.* 2015b; Okino *et al.* 2013) and immune responses (Kameka *et al.* 2014; Okino *et al.* 2017; Okino *et al.* 2014; Wang *et al.* 2006) were restricted and relied only on trachea. The innate immune response is the first line of host defence against pathogens and consists of a network of mechanisms, molecules, and cells, particularly pattern recognition receptors (PRRs), which function to recognise various pathogen-associated molecular patterns (PAMPs) (He *et al.* 2016). PAMPs are typically conserved between pathogens and can be recognized by the host cell through membrane-associated and intracellular toll-like receptors (TLRs). Both TLRs and cytosolic RIG-I-like receptors (RLRs) are essential PRRs involved in recognition of viral particles, and TLR3 is well known for the recognition of RNA virus-encoded PAMPs (Bauer *et al.* 2008). Host TLR3 recognises and binds to double-stranded (ds) RNA intermediates produced during viral replication (Alexopoulou *et al.* 2001). Melanoma differentiation-associated protein 5 (MDA5) detects long-duplex RNAs or dsRNA of positive-strand viruses (Wu *et al.* 2013), and cytoplasmic viral RNA, to induce antiviral immune response such as interferon production (Yu *et al.* 2017). The stimulation of TLRs leads to the transcription of genes encoding type I interferons such as interferon-alpha (IFN-α) and interferon-beta (IFN-β). These interferons are secreted from the infected cell, signalling the transcription of antiviral effector genes, collectively called interferon-stimulated genes (ISGs) (Kawai & Akira 2006). Chickens challenged with an attenuated IBV strain (H120) showed an increased expression of pro-inflammatory cytokine genes in their tracheal tissues by recruiting polymorph and mononuclear leukocytes to the primary site of infection (Guo *et al.* 2008). Okino *et al.* (2014) further reported that the expression of pro-

inflammatory cytokines such as interleukin-6 (IL-6) is involved in the activation of innate immune responses in the early phase of IBV infection.

As the underlying immunity induced by vaccination is responsible for protection, it would be appropriate to identify underlying immune mediators as potential quantitative indicators. With an understanding of the kinetics of the challenge virus and early immune responses in susceptible (naïve) birds (Chapter 3), we proceeded to challenge IBV-vaccinated birds and then cross-compare results against the vaccinated-unchallenged. Despite advances in the use of molecular and immunological tools in poultry research, demonstration of vaccine protection efficacy outlined by the European Pharmacopoeia, has remained the same for decades and heavily relies on subjective cilia-stopping readings. With the aim of introducing quantitative IBV protection indicators, this study reports on IBV growth kinetics, early mucosal and cell-mediated immune responses, and gene signatures in head- associated lymphoid and respiratory tissues in vaccinated and vaccinated IBV M41 challenged chickens.

4.2 Material and methods

4.2.1 Chicks

Ninety, day-old broiler chicks were obtained from a commercial hatchery and kept until 26- day-old according to animal welfare guidelines, under strict biosecurity measures in an isolation unit at the University of Liverpool (Chapter 2.3). Chicks were reared on deep litter with antibiotic-free water and feed provided *ad libitum* (Chapter 2.4).

4.2.2 IBV vaccines

Two commercial live IBV vaccines were used in this study, Massachusetts serotype (H120 ($10^{3.5}$ EID_{50}/ml)) and 793B serotype (CR88 ($10^{4.25}$ EID_{50}/ml)). Vaccines were stored at 4 °C until required, and then dissolved in sterile distilled water (SDW)

according to the manufacturer's instructions (Boehringer Ingelheim Animal Health Limited, UK). For combined vaccination, one vial of each live vaccine was mixed after preparation and retained on crushed ice. Each chick received 100 µl of vaccine or SDW via the ocular (50 µl) and nasal (50 µl) route according to the experimental design.

4.2.3 IBV challenge strains

A virulent strain of IBV M41 was used as the challenge virus. This virus was propagated and titrated in 10 days old SPF ECE at 37 °C (Chapter 2.1). The eggs were inoculated via the allantoic cavity as described previously (Bijlenga *et al.* 2004). Virus rich allantoic fluid was collected and titrated ($10^{5.75}$ CD_{50}/bird) in chicken tracheal organ cultures (TOCs) (Chapter 2.2), and confirmed to be free from other viruses, bacterial or fungal contaminants (Chapter 2.6).

4.2.4 Experimental design

A total of 90 day-old chicks were divided into two initial groups: unvaccinated (n=30) and vaccinated (n=60). Chicks in the unvaccinated group were sham-inoculated with 0.1 ml of chilled vaccine-free SDW, while the vaccinated group received 0.1 ml of the combined Mass and 793B vaccine, both via the oculonasal route. At 21 days post vaccination (dpv), the vaccinated group was further divided into two groups: vaccinated-unchallenged (n=30) and vaccinated-challenged (n=30). To assess the viral load and immune responses in vaccinated chicks, birds received either 0.1 ml of virus-free allantoic fluid (vaccinated-unchallenged) or 0.1 ml of $10^{5.75}$ CD_{50}/bird of virulent M41 virus (vaccinated-challenged) via the oculonasal route. Unvaccinated chicks received 0.1 ml of virus-free allantoic fluid via the same route and were kept as a control (unvaccinated-unchallenged). Clinical signs were recorded daily, and at 1-5 days post challenge (dpc), five chicks from each group were sampled. Oropharyngeal

(OP) and cloacal swabs (CL) were collected for virus detection by RT-PCR and viral load and quantification by qRT-PCR. Lachrymal fluid was collected to assay for anti-IBV antibodies by monoclonal ELISA, and blood was collected via the brachial vein, with the serum separated and stored at - 20 °C. The five sampled birds from each group were then humanely euthanized using sodium pentobarbitone. Harderian gland (HG), choanal cleft, turbinate, pharyngeal tissue and trachea samples were collected and stored at -20 °C in RNA*Later* (Qiagen, Crawley, UK) for determination of viral load and host gene expression analysis by quantitative real-time RT- PCR (qRT-PCR). Tracheal portions were also collected in 10% buffered formalin and examined by histopathology. Ciliostasis testing was conducted on tracheal rings at the point of sampling using a light microscope.

4.2.5 Evaluation of tracheal health (ciliostasis)

Tracheas were extracted from each bird during necropsy and processed for ciliary protection scoring as previously described (Awad *et al.* 2016c; Cook *et al.* 1999; de Wit *et al.* 2011b). According to the PhEur, individual birds yielding 50% or more tracheal explants with ciliary activity were considered to have been protected by vaccine against the challenge virus (Council of Europe 2007).

4.2.6 Swab samples

Five OP and CL swabs were taken from each group at all sampling time points. Swabs from the same group were pooled, dipped into 1.5 ml of TOC medium, then stored at -70 °C until required (Chapter2.8.1).

4.2.7 Histopathology

For histopathology examination, the proximal trachea was fixed in 10% buffered formalin for 24-48 hours and then routinely processed and embedded in paraffin for

histological examination; several parameters were scored 0 – 3 as previously described (Chen *et al.* 1996; Chhabra 2016b) (Chapter 2.11).

4.2.8 RNA extraction from swabs and tissues

4.2.8.1 Extraction of RNA (swab)

RNA was extracted from OP and CL swabs using the QIAamp viral RNA mini kit (Qiagen, UK) following the manufacturer's instructions (Chapter 2.12.2).

4.2.8.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Following extraction, RT-PCR was conducted on swabs to detect a partial IBV S1 sequence (393bp) as previously described (Cavanagh *et al.* 1999; Ganapathy *et al.* 2015) (Chapter 2.14).

4.2.8.3 Extraction of RNA (tissue)

Total RNA was extracted from all collected tissues using the RNeasy Plus Mini Kit (Qiagen) according to the manufacturer's instructions (Chapter 2.13).

4.2.9 IBV Quantitative reverse transcription PCR (qRT-PCR)

Viral load was quantified from RNA extracted from the HG, choanal cleft, turbinate, pharyngeal tissue, trachea and OP and CL swabs (Ball *et al.* 2016b; Chhabra *et al.* 2018; Jones *et al.* 2011b) (Chapter 2.20).

4.2.10 Host gene expression

Host gene expression analysis was carried out for samples collected at 1-5 dpc. QRT-PCR of cDNA samples was performed in triplicate using LightCycler 480 SYBR Green I Master mix and primers (Chapter 2.21, Table 2.5). Data from qRT-PCR were normalised against 18S ribosomal RNA expression using a relative standard curve method (Chhabra *et al.* 2018; Kuchipudi *et al.* 2012; Okino *et al.* 2013; Rajesh *et al.* 2016) and the data

were presented as fold change difference in gene expression of virus- versus unvaccinated-unchallenged group.

4.2.11 Serum for humoral antibody detection

Blood samples from the brachial vein were obtained from five birds of each group at 5 dpc and placed in labelled 5 ml bijous without coagulant overnight at room temperature. Serum was stored at -20 °C until further use (Chapter 2.8.2).

4.2.12 Lachrymal fluid for mucosal antibody detection

Lachrymal fluid was collected at 1-5 dpc, using sodium chloride and immediately centrifuged at 3000 x g for 5 mins, and the supernatant was stored at -20 °C as previously described (Ganapathy *et al.* 2005) (Chapter 2.8.3).

4.2.13 Partial purification of IBV antigen and quantification using the Bradford Assay

IBV M41 was propagated in 10 days old SPF ECE. Partial purification of the antigen was carried out by adding 25 ml of allantoic fluid and centrifuging the liquid at 1,150 × g, 4 °C for 10 mins. The pellets were washed, re-suspended in PBS and filtered through 0.22 µm filter. The virus suspensions were overlaid onto 30% sucrose and ultra-centrifuged at 102,400 × g, 4 °C for 4 h. Pellets were stored at -70 °C until use (Ganapathy *et al.* 2005; Keep *et al.* 2015) (Chapter 2.16). The total concentration of purified protein was quantified by using the 5x Bradford assay, with 1:10 serial dilutions of bovine serum albumin (BSA). The purified antigen protein concentration was calculated after comparing the optical density (OD) to the BSA standard curve (Chapter 2.17).

4.2.14 Serology

4.2.14.1 ELISA detection of IBV antibodies

A commercial IBV ELISA kit (IDEXX, Westbrook, Maine, USA) was used to determine anti-IBV antibodies for all groups according to the manufacturer's guidelines, with a positive ELISA titre cut-off of 396 (Chapter 2.18.1).

4.2.14.2 Measurement of local antibodies against IBV

Lachrymal fluid for IBV-specific IgA and IgY at 1-5 dpc were assayed by using an indirect ELISA (Dhinakar Raj & Jones 1996c; Fournier-Caruana *et al.* 2003; Ganapathy *et al.* 2005; Mockett & Cook 1986) (Chapter 2.18.2).

4.2.15 Statistical analysis

Data were confirmed to be normally distributed and analysed using the student's t-test (two groups) or a one-way analysis of variance (ANOVA), followed by the post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00. Differences between groups were considered significant at $P<0.05$ (chapter 2.22).

4.3 Results

4.3.1 Clinical signs and gross lesions

No clinical signs or gross lesions were seen in birds from the control group (unvaccinated-unchallenged) or from the vaccinated-unchallenged group. In the vaccinated-challenged group, clinical respiratory signs were seen after 3 dpc, which consisted of head shaking, scratching, sneezing and restlessness.

4.3.2 Ciliostasis

Protection of ciliary activity in the trachea was measured at 1-5 dpc, and the vaccinated-challenged group showed average ciliary protection of ≥ 93.5 %, whereas protection in the vaccinated-unchallenged group remained above 95% (Figure 4.1).

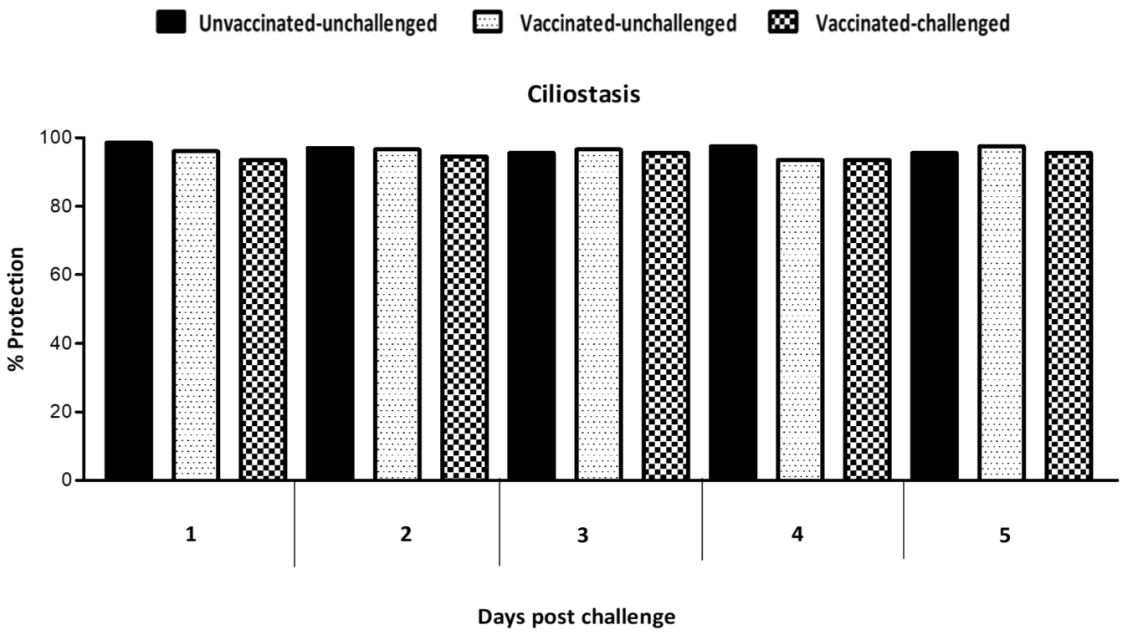

Figure 4.1. Tracheal ciliary activity in the unvaccinated and vaccinated chickens in the period of 1-5 dpc.

4.3.3 Histological changes

Sections showing intact cilia, normal tracheal epithelia and goblet cells were seen in the control group (Figure 4.2 A).

Tracheal damage was observed in the vaccinated-unchallenged group, which consisted of ciliary loss, a decrease in the number of mucous cells and mild lymphoid infiltration (Figure 4.2 B and C). Histopathological scores were significantly higher at all-time points compared to unvaccinated-unchallenged group (Figure 4.3 A), and at 1 dpc compared to later time points (Figure 4.3 B), with a mean score of 8.6±0.66 (Table 4.2).

In the vaccinated-challenged group, tracheal lesions were moderate. These lesions included decreased mucous cells, epithelial degeneration and lymphoid infiltrations at 1 dpc (Figure 4.2 D-F). However, tracheal histopathology scores were significantly

higher in the vaccinated-challenge group than those in the vaccinated-unchallenged birds at 5 dpc, and at all days compared to unvaccinated-unchallenged birds (Figure 4.3 A), lesion severity peaked by 1 dpc with a mean score of 7 ± 1 (Table 4.3).

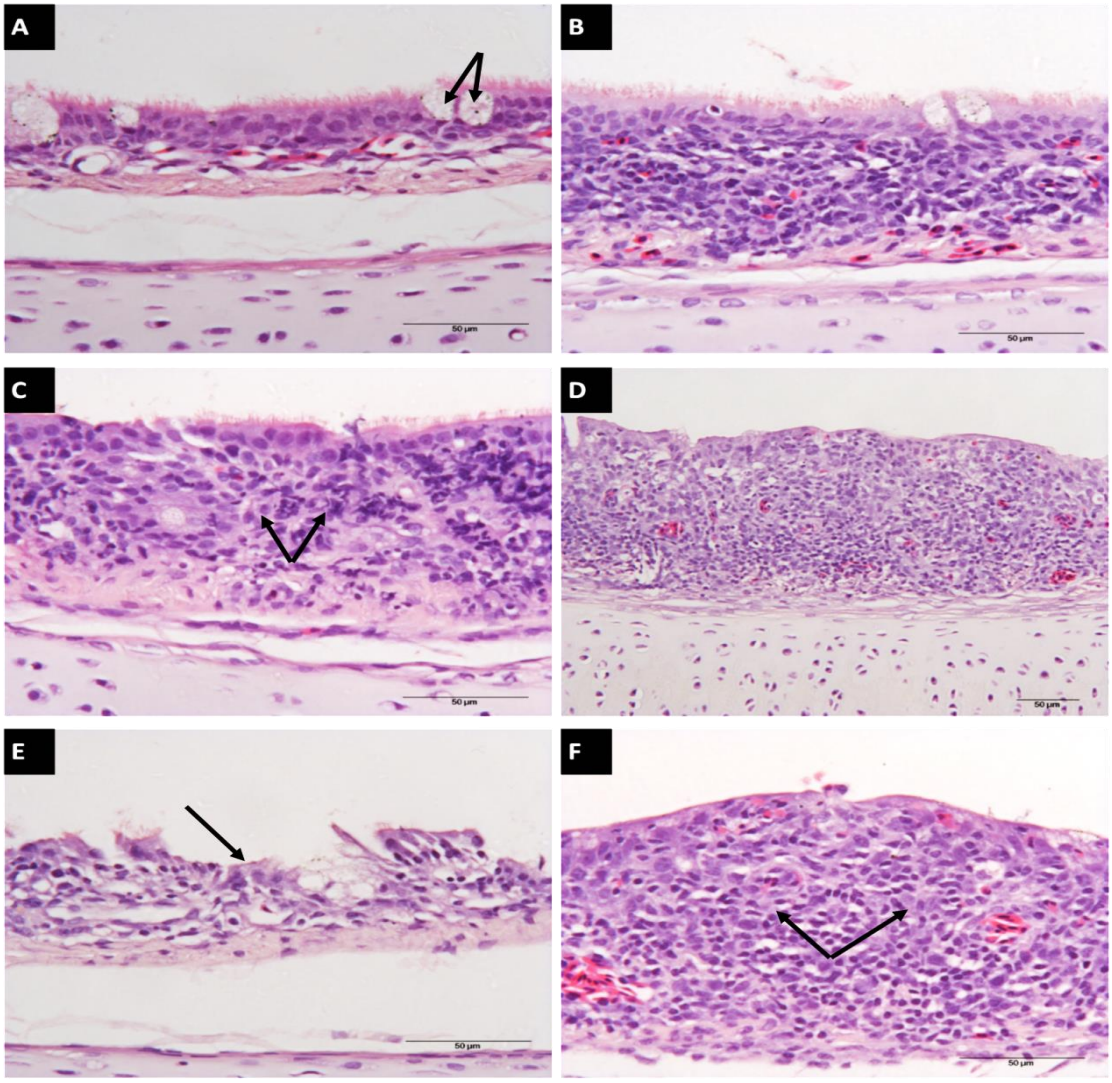

Figure 4.2. Arrows showing histopathological findings in the trachea. 400x magnification; haematoxylin and eosin (H&E) stain. (A) Unvaccinated-unchallenged control group (score; 0) showing intact cilia and goblet cells. (B and C) Vaccinated group (score; 1) showing mild loss of cilia with decreased mucous cells (B) and lymphoid infiltration of the lamina propria (C). (D-F) Vaccinated-challenged group (score; 2) showing a decrease in mucous cells (D), epithelial degeneration (E) and moderate infiltration by lymphocytes plasma cells and heterophils (F; magnification 100x).

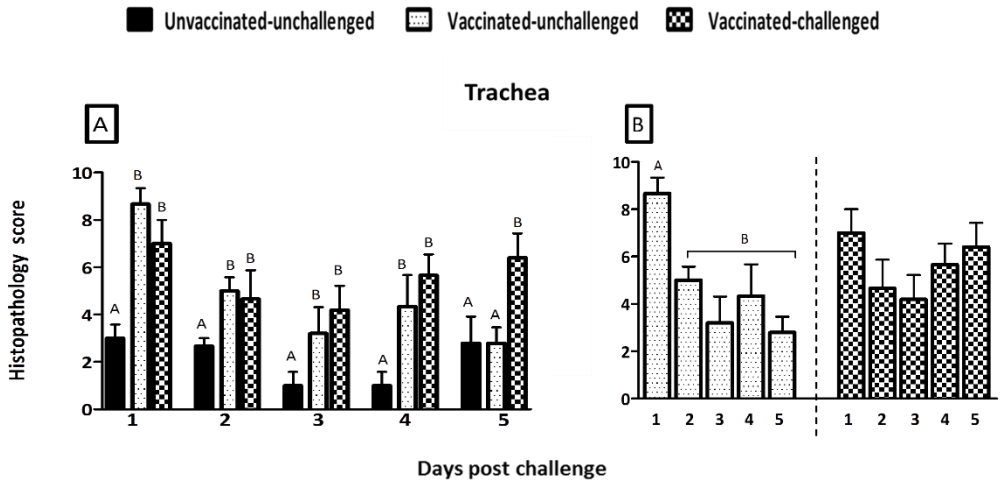

Figure 4.3. Histopathological score of lesions in the trachea of vaccinated-unchallenged versus vaccinated-challenged groups (A). Comparison between sampling days of the vaccinated birds on the same group (B). Birds were vaccinated or vaccinated and challenged with IBV M41 at a dose of $10^{5.75}$ CD_{50}/bird. Data are presented as the average mean of total scores for each parameter with standard error bars. One trachea section was scored/bird. Significant differences between the groups indicated with different letters ($P<0.05$).

Table 4.1. Histopathology of the trachea in unvaccinated-unchallenged broiler chicks. The scores for each histopathological criterion in each bird were added together and the mean score for the birds at each timepoint was calculated (Chen *et al.* 1996). The mean histopathological lesion score is expressed as ± SEM. (1, 2 and 4 dpc n =3; 3 and 5 dpc n=5).

Days post challenge	Bird No.	Epithelial deciliation	Epithelial degeneration	Decrease mucous cells	Heterophil infiltration	Epithelial hyperplasia	Lymphoid infiltration	Total score	Mean score ± SEM
1	**1**	1	1	0	0	0	0	2	**3 ± 0.57**
	2	1	1	0	0	1	1	4	
	3	0	0	0	0	1	1	3	
2	**1**	0	1	0	0	1	1	3	**2.6 ± 0.33**
	2	1	1	0	0	0	0	2	
	3	1	1	1	0	0	0	3	
3	**1**	0	0	0	0	0	0	0	**0.8 ± 0.37**
	2	1	1	0	0	0	0	2	
	3	0	1	0	0	0	0	1	
	4	0	0	0	0	0	0	0	
	5	0	0	0	0	0	1	1	
4	**1**	0	0	0	0	0	1	1	**1± 1.57**
	2	1	1	0	0	0	0	2	
	3	0	0	0	0	0	0	0	
5	**1**	0	0	0	0	0	1	1	**2.8± 1.11**
	2	0	0	0	0	0	1	1	
	3	0	1	0	0	1	1	3	
	4	0	0	0	0	1	1	2	
	5	1	2	1	1	1	1	7	

Table 4.2. Histopathology of the trachea in broiler chicks vaccinated with IBV (H120+CR88) strains, and mock-challenged. The scores for each histopathological criterion in each bird were added together and the mean score for the birds at each time point was calculated. The mean histopathological lesion score is expressed as ± SEM. (1, 2 and 4 dpc n =3; 3 and 5 dpc n=5).

Days post challenge	Bird No.	Epithelial deciliation	Epithelial degeneration	Decrease mucous cells	Heterophil infiltration	Epithelial hyperplasia	Lymphoid infiltration	Total score	Mean score ± SEM
1	1	2	2	2	0	0	2	8	8.6 ± 0.6
	2	2	1	2	1	2	2	10	
	3	1	1	2	2	1	1	8	
2	1	1	1	1	0	0	2	5	5 ± 0.5
	2	1	1	1	0	0	1	4	
	3	1	2	0	0	1	2	6	
3	1	0	0	0	0	0	1	1	3.2 ± 0.1
	2	0	0	0	0	0	1	1	
	3	1	1	0	0	0	1	3	
	4	1	1	1	0	0	1	4	
	5	2	2	1	1	0	1	7	
4	1	0	0	2	0	0	1	3	4.3 ± 1.3
	2	0	0	1	0	0	2	3	
	3	1	1	2	1	0	2	7	
5	1	0	1	0	0	1	1	3	2.8 ± 0.6
	2	0	0	0	0	2	1	3	
	3	1	1	0	0	1	2	5	
	4	0	0	0	0	0	1	1	
	5	0	1	0	0	0	1	2	

Table 4.3. Histopathology of the trachea in broiler chicks vaccinated with IBV (H120+CR88) strains, and challenged with M41. The scores for each histopathological criterion in each bird were added together and the mean score for the birds at each timepoint was calculated. The mean histopathological lesion score is expressed as ± SEM. (1 dpc n=2, 2 and 4 dpc n =3; 3 and 5 dpc n=5).

Days post challenge	Bird No.	Epithelial deciliation	Epithelial degeneration	Decrease mucous cells	Heterophil infiltration	Epithelial hyperplasia	Lymphoid infiltration	Total score	Mean score ± SEM
1	**1**	1	1	1	1	1	1	6	**7 ± 1**
	2	1	1	2	1	1	2	8	
2	**1**	1	2	1	1	0	2	7	**4.6 ± 1.2**
	2	1	1	0	0	0	1	3	
	3	1	1	0	1	0	1	4	
3	**1**	0	0	0	0	1	1	2	**4.2 ± 1.0**
	2	1	1	0	0	1	1	4	
	3	1	1	1	0	1	2	6	
	4	0	1	0	0	0	1	2	
	5	2	2	1	0	1	1	7	
4	**1**	1	1	1	0	0	1	4	**5.6 ± 0.8**
	2	2	2	1	0	0	1	6	
	3	2	1	2	1	0	1	7	
5	**1**	1	2	1	0	1	1	6	**6.4±1.02**
	2	1	2	2	0	2	1	7	
	3	0	1	2	0	1	1	5	
	4	0	1	1	0	1	1	4	
	5	1	2	2	1	2	2	10	

4.3.4 Detection of IBV from OP and CL swabs by RT-PCR

Throughout the study period, no virus was detected in the unvaccinated-unchallenged control group. The viral genome was detected by RT-PCR in the pooled OP and CL swabs from both vaccinated groups all-time points (1–5 dpc).

4.3.5 Systemic humoral immune responses by ELISA

High levels of IBV maternal-derived antibody (MDA) were detected in the day-old control birds, with the mean antibody titre being 1376±386. At 21 dpv, antibody titres had declined and neither the unvaccinated nor the vaccinated group (204 and 333 respectively) had titres above the positive cut-off point (396). At 5 dpc, significant antibody titres were observed in the vaccinated-unchallenged and vaccinated-challenged groups (580.5 and 986.4) compared to the control group (21). However, there was a significant difference between the vaccinated groups (Figure 4.4).

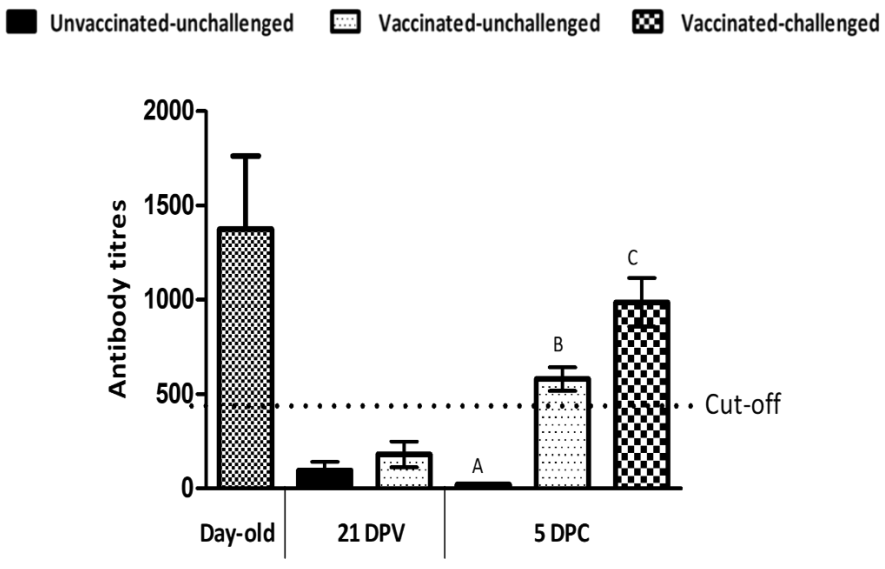

Figure 4.4. Mean anti-IBV ELISA antibody titres in the vaccinated (H120+CR88) and vaccinated M41-challenged chicks at 5 dpc. The ELISA cut-off titre is 396.

4.3.6 Local humoral immune responses (IgA and IgY) by indirect ELISA

4.5.6.1 Specific IgA against IBV

The level of specific IgA anti-IBV in lachrymal fluids in vaccinated birds was seen at 21 dpv (1.179±0.239). Titres then declined in the vaccinated-unchallenged group until 5 dpc (26 dpv) (0.901±0.150). For chickens in the vaccinated-challenged group, a positive IgA titre was witnessed at 24 hours post-challenge, which peaked at 3 dpc. Moreover, a significant increase was observed in the IgA levels in the vaccinated-challenged chickens at 3-5 dpc compared to the vaccinated birds (Figure 4.5 A). The IgA titres from lachrymal fluid showed a statistical increase among the vaccinated-challenged group at 3, 4 and 5 dpc compared to 1 and 2 dpc.

4.5.6.2 Specific IgY against IBV

The titre of specific IgY anti-IBV in vaccinated birds was detected at 21 dpv (0.269±0.045). This titre then decreased until 26 dpv (0.203±0.015) in the vaccinated-unchallenged group, while in the vaccinated-challenged birds, IgY levels gradually increased until 5 dpc. There was a significantly higher IgY level in the vaccinated-challenged chickens at 3-5 dpc compared to the vaccinated birds (Figure 4.5 B). Similar to the IgA titres, a significant increase was observed in the vaccinated-challenged group at 3, 4 and 5 dpc compared to 1 and 2 dpc.

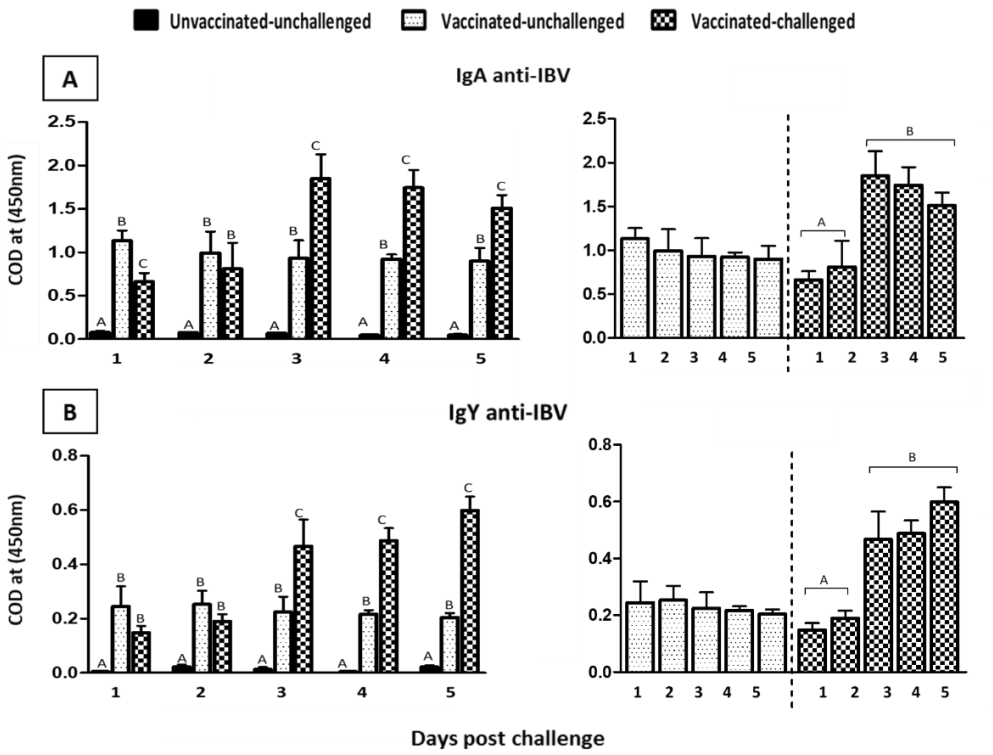

Figure 4.5. Measurement of IBV-specific (A) IgA and (B) IgY titres using an indirect ELISA from lachrymal fluid. Mean IgA and IgY from lachrymal fluids collected at 1-5 dpc (n=5), data expressed as mean of corrected optical density (COD). Significant differences are indicated with different letters.

4.3.7 Viral RNA quantification

4.3.7.1 Pooled swabs

Viral RNA load in the unvaccinated-unchallenged group were below the detection limit on all days post challenge (Figure 4.6). The viral RNA load in OP swabs from vaccinated-unchallenged birds increased after 1 dpc and peaked at 5 dpc (2.64 log REU), while the viral RNA load in vaccinated-challenged birds peaked earlier at 3 dpc (2.18 log REU). The vaccinated-unchallenged birds had a greater IBV concentration at all-time points compared to the vaccinated-challenged group. Viral RNA load of the CL swabs in vaccinated-unchallenged birds peaked at 4 dpc (1.56 log REU), while the viral RNA load in vaccinated-challenged birds peaked earlier at 2dpc (2.27 log REU). In contrast to the

OP samples, the viral load in the vaccinated group was lower at 1, 2 and 5 dpc when compared to the vaccinated-challenged group.

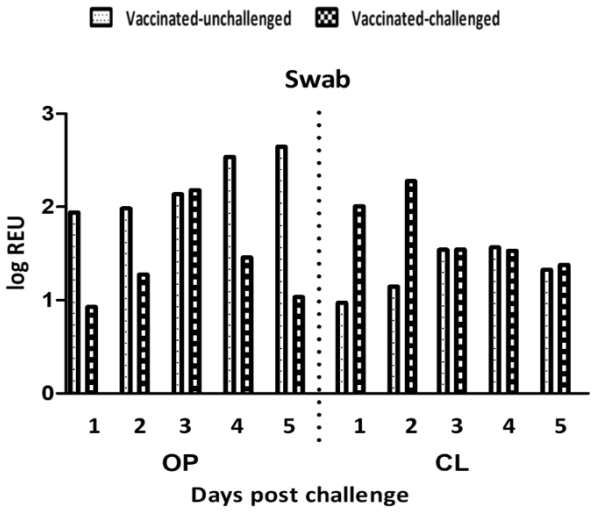

Figure 4.6. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA. IBV loads at 1, 2, 3, 4 and 5 dpc in OP and CL swabs respectively.

4.3.7.2 Tissues

The unvaccinated-unchallenged group have already been screened for viral RNA in Chapter 3 and all were negative. Viral RNA loads in the HG, choanal cleft, turbinate and pharyngeal tissues samples were higher in the vaccinated-unchallenged birds at all days post challenge compared to the vaccinated-challenged group (Figure 4.7), with the exception of 3 dpc in the HG, 2 dpc in pharyngeal tissue and 2-5 dpc for the turbinate. Viral RNA loads in vaccinated-unchallenged birds peaked at 1 dpc in the turbinate, choanal cleft and pharyngeal tissue (2.60 log REU, 2.38 log REU and 2.79 log REU respectively), and 2 dpc in HG (3.65 log REU). There is a significant higher difference at 1 dpc in all tissues, except the turbinate and trachea in the vaccinated-

unchallenged compared to vaccinated-challenge groups (Figure 4.7 A, B, C and E). The tracheal viral load was significantly higher at 1 to 4 dpc in vaccinated-challenge birds compared to the vaccinated-unchallenged groups (Figure 4.7 D).

In the vaccinated-challenge group, viral RNA levels peaked at 2 dpc in turbinate, choanal cleft and pharyngeal tissue (2.03 log REU, 1.11 log REU and 1.63 log REU respectively), while in the HG and trachea, viral RNA loads peaked at 3 dpc (3.52 log REU and 3.91 log REU respectively) (Figure 4.7 A-E). The unvaccinated-challenged data is included in Chapter 3.

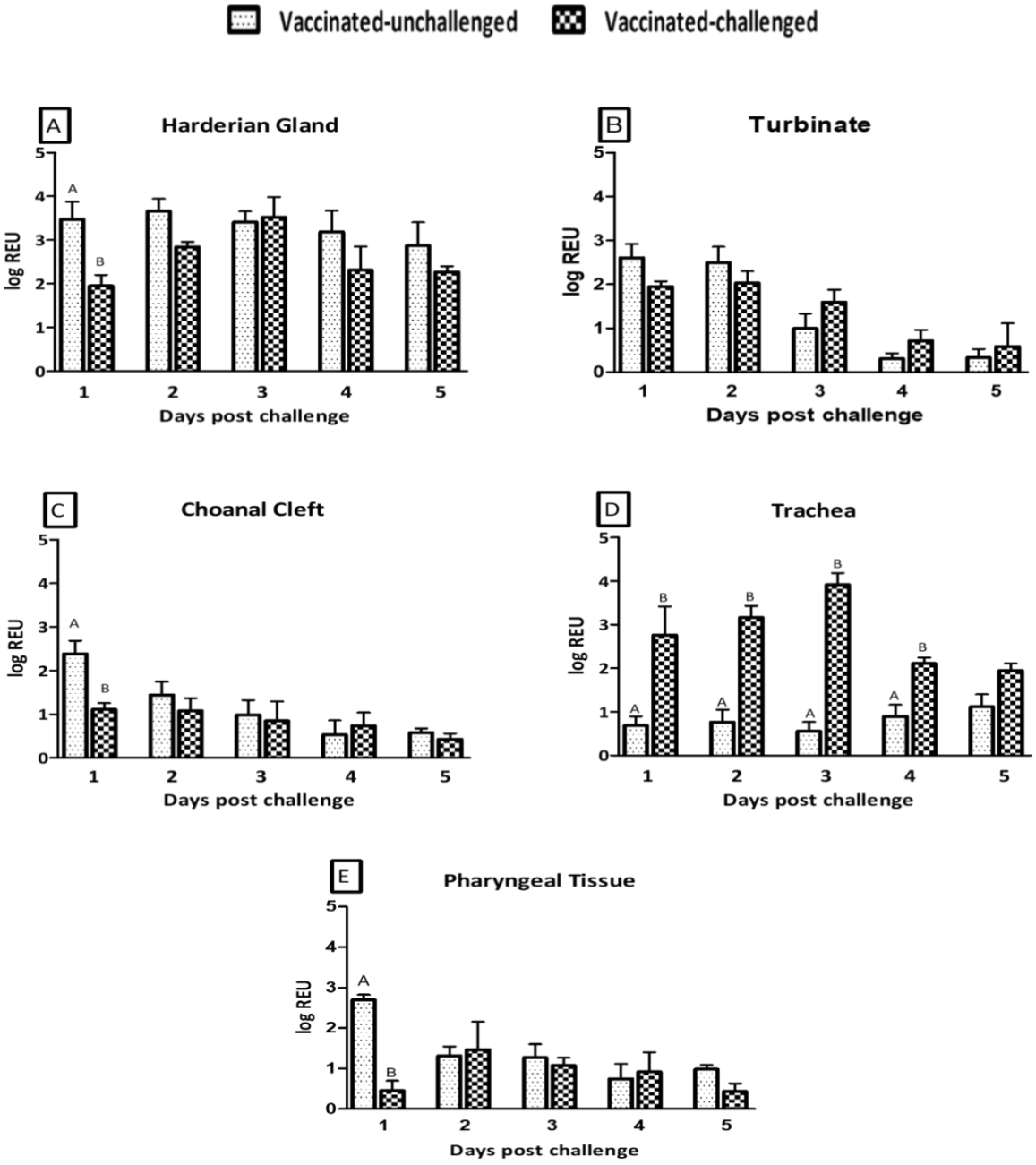

Figure 4.7. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA in (A) Harderian gland, (B) turbinate, (C) choanal cleft, (D) trachea and (E) pharyngeal tissue. Significant differences between the groups are indicated with different letters (P<0.05).

4.3.8. Expression of host TLR3 and MDA5 mRNA

Harderian gland (HG)

Significant up-regulation in the mRNA expression of TLR3 in vaccinated-unchallenged birds, was observed at 2 dpc. In vaccinated-challenged birds, significant up-regulation was present for the first two days when compared with the control or vaccinated-unchallenged group (Figure 4.8 A). Expression of MDA5 showed significant up-regulation at 1 and 2 dpc in vaccinated-challenged birds compared with control or vaccinated-unchallenged group. (Figure 4.9 A).

Choanal cleft

There was only significant up-regulation in the mRNA expression of TLR3 at 1 dpc in vaccinated and vaccinated-challenged birds compared to the control. Significant changes were noted at 1 dpc in the vaccinated-challenged birds compared to the vaccinated-unchallenged groups (Figure4.8 C). The same was true for mRNA expression of MDA5, with significant up-regulation only at 1 dpc in vaccinated and vaccinated-challenged birds compared to the control group (Figure 4.9 C).

Turbinate

Similar to the choanal cleft, there was only significant up-regulation in the mRNA expression of TLR3 at 1 dpc in the vaccinated-challenged birds when compared with the control and vaccinated-unchallenged group (Figure 3.8 B). For mRNA expression of MDA5, results indicated significant up-regulation at 1 and 2 dpc of both vaccinated and vaccinated-challenged birds compared to the control group. In addition, a significant high difference was also noticed in the vaccinated-challenged birds compared to the vaccinated-unchallenged at 1 dpc (Figure 3.9 B).

Trachea

Significant up-regulation was noted in the mRNA expression of TLR3 at 1 and 2 dpc in the trachea for vaccinated-unchallenged birds, and at 1 dpc in the vaccinated-challenged birds, when compared to the control group (Figure 3.8 D). There was significant up-regulation of MDA5 gene expression at 2 dpc in vaccinated-unchallenged birds, and at 1-3 dpc in vaccinated-challenged birds, compared with the control group. Significant differences were observed between vaccinated and vaccinated-challenged groups at 1 and 3 dpc (Figure 3.9 D).

Pharyngeal tissue

A significant down-regulation was noted in the mRNA expression of TLR3 at 3 dpc in vaccinated-unchallenged birds, and the significant up-regulation of this gene was observed at 1 dpc compared to the control group (Figure 4.8 E). In the mRNA expression of MDA5, there was a significant up-regulation at 2 dpc in the vaccinated-unchallenged group and a significant up-regulation in the mRNA expression of this gene at 1 dpc in the vaccinated-challenged compared to the control group. A significant difference was observed between vaccinated and vaccinated-challenged groups at 1 and 2 dpc (Figure 4.9 E).

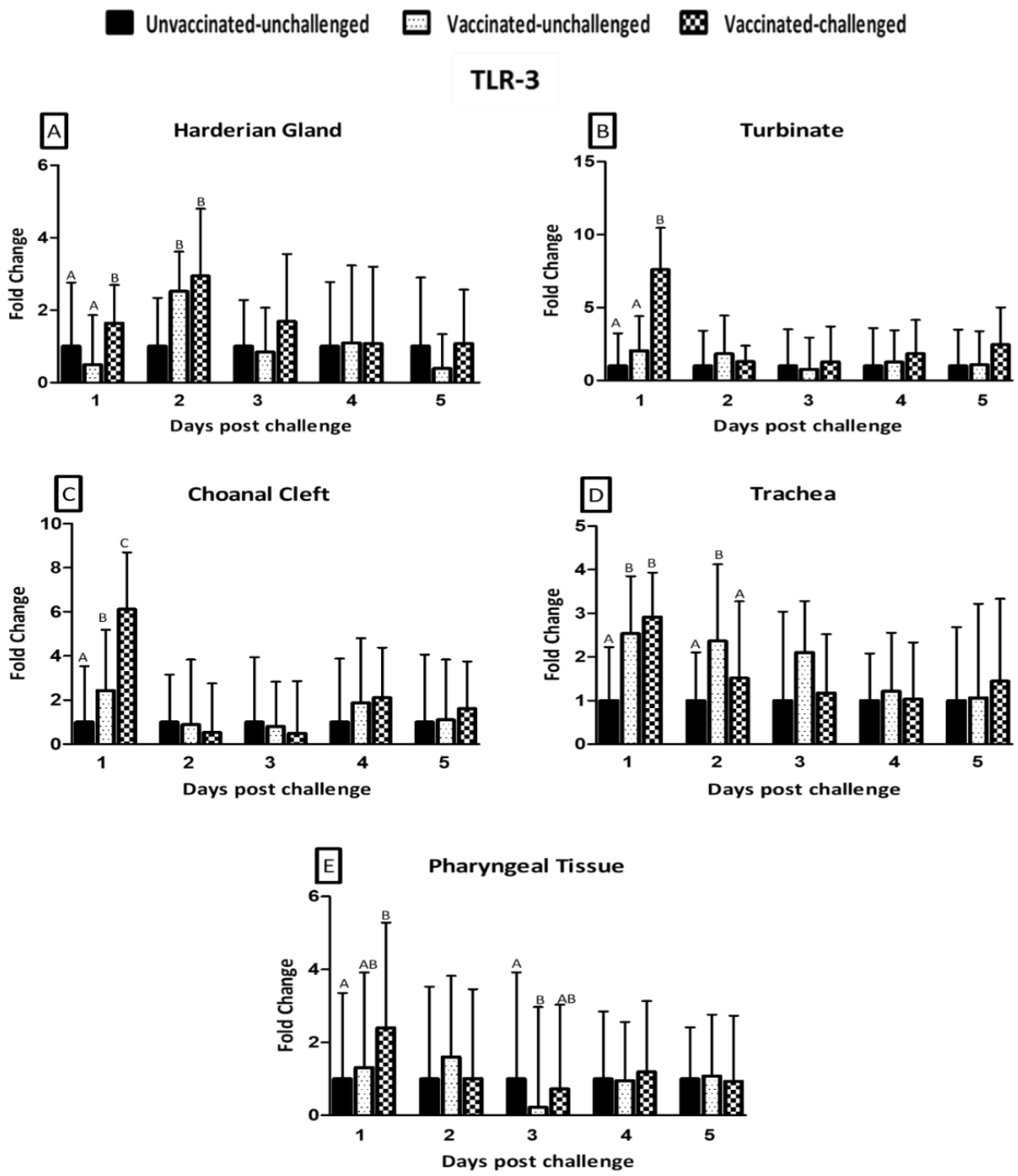

Figure 4.8. Relative mRNA expression of TLR3 (A-E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue sample of chickens. Data represents the mean (n=5), with error bars indicating standard errors, and are expressed as fold change relative to the unvaccinated-unchallenged control group. Significant differences between the groups are indicated with different letters ($P<0.05$). NO significant differences between the other groups indicated with letter AB.

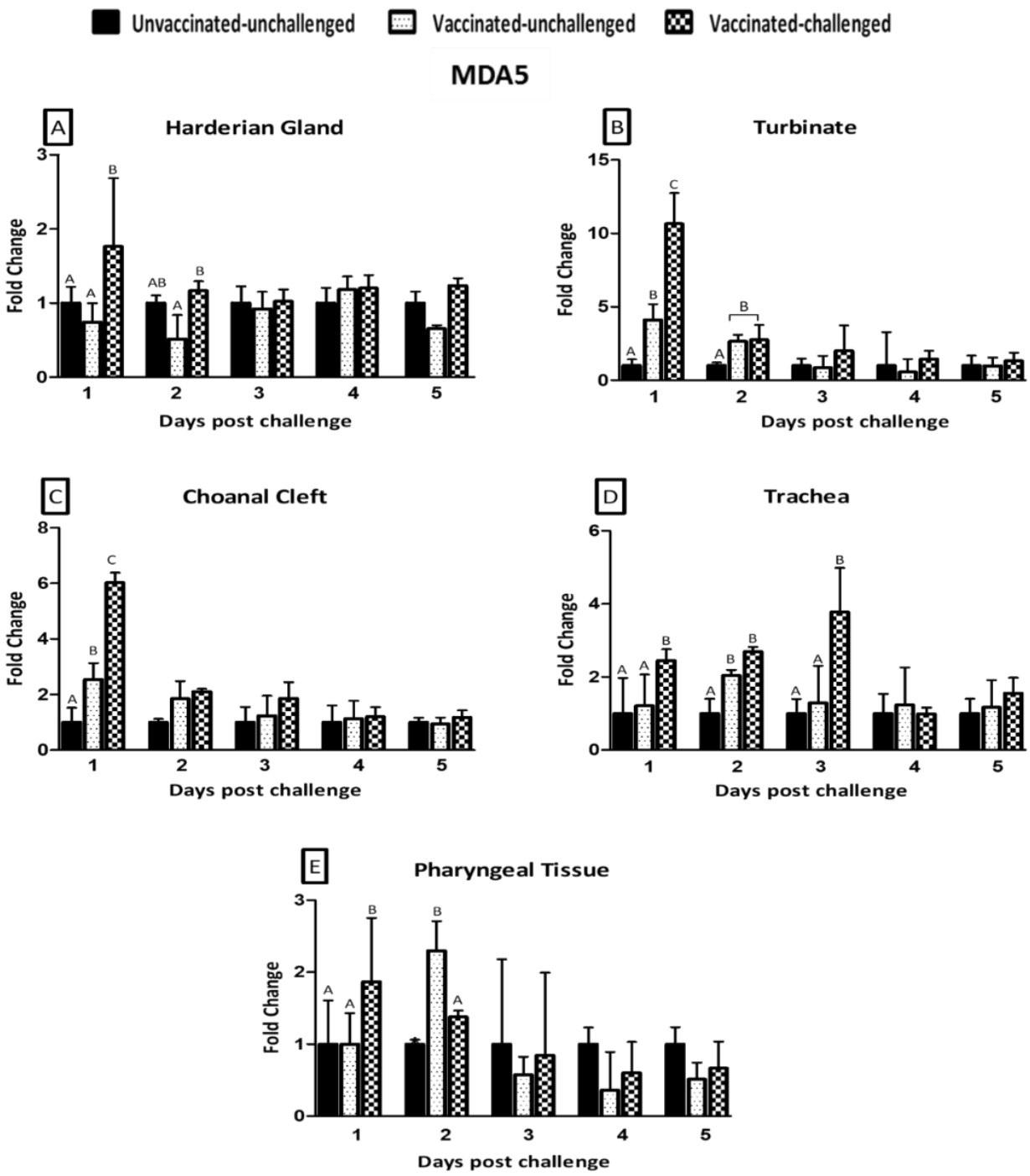

Figure 4.9. Relative mRNA expression of MDA5 (A-E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue sample of chickens. Data represents the mean (n=5), with error bars indicating standard errors, and are expressed as fold change relative to the unvaccinated-unchallenged control group. Significant differences between the groups indicated with different letters ($P<0.05$). NO significant differences between the other groups indicated with letter AB.

4.3.9 Expression of host IFN-α and IFN-β mRNA

Harderian gland

Significant down-regulation of INF-α was seen in all sampling days in both vaccinated and vaccinated-challenged birds compared to the control group. There was only a significant difference at 3 dpc between vaccinated-challenged birds and the vaccinated-unchallenged group (Figure 4.10 A). There was significant up-regulation in IFN-β mRNA expression at 3 and 4 dpc in vaccinated-challenged birds when compared with the vaccinated-unchallenged and control groups (Figure 4.11 A).

Choanal cleft

There was only significant up-regulation in the mRNA expression of IFN-α at 1 dpc in the vaccinated-challenged birds when compared with the vaccinated-unchallenged and control group. No other changes were observed in any sampling days after one dpc (Figure 4.10 C). There was significant down-regulation of IFN-β at 1 dpc in both vaccinated groups when compared with the control group, followed by up-regulation at 4 dpc in both groups and 5 dpc only in the vaccinated-challenge group (Figure 4.11 C).

Turbinate

Significant up-regulation of IFN-α mRNA was observed in both vaccinated and vaccinated-challenged birds at 1 dpc. Significant differences were observed high in the vaccinated-challenged birds compared to vaccinated-unchallenged bird at the same time point (Figure 4.10 B). There was significant down-regulation of IFN-β in both vaccinated groups at 1 dpc compared to the control group, with the vaccinated-unchallenged group also being down-regulated at 5 dpc. There was significant up-

regulation in mRNA expression of IFN-β at 4 dpc in both vaccinated groups compared with the control group (Figure 4.11 B).

Trachea

There was only significant up-regulation in IFN-α expression at 1 dpc in the vaccinated-challenged birds compared to the vaccinated-unchallenged and control group (Figure 4.10 D). Expression of IFN-β mRNA was significantly down-regulated in both vaccinated groups at 1 dpc when compared with the control group. However, at 5 dpc, expression was significantly up-regulated at 5 dpc in both vaccinated and vaccinated-challenged groups compared with the control group (Figure 4.11 D).

Pharyngeal tissue

There was a significant down-regulation in the mRNA expression of IFN-α at 1 and 3 dpc in both vaccinated and vaccinated-challenged birds when the tissue was compared with tissue from the control group. There were no significant differences between vaccinated and vaccinated-challenged groups at I and 3 dpc (Figure 4.10 E). Expression of IFN-β mRNA was significantly down-regulated in both vaccinated groups at 1 dpc when compared with the control group. There was a significant up-regulation of this gene at 3 dpc in vaccinated-challenged groups and at 4 dpc in both groups (Figure 4.11 E).

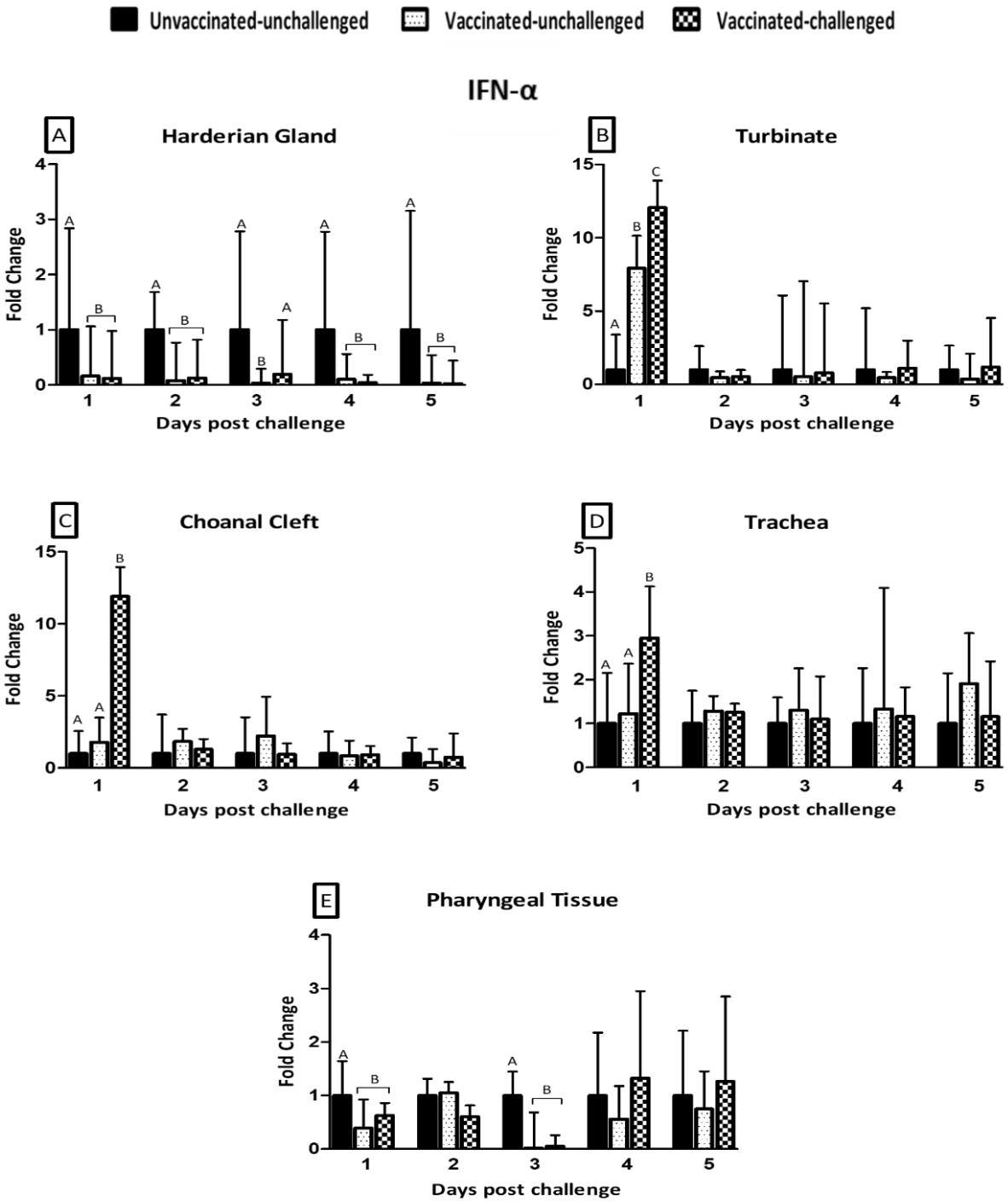

Figure 4.10. Relative mRNA expression of IFN-α (A-E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue sample of chickens. Data represents the mean (n=5), with error bars indicating standard errors, and are expressed as fold change relative to the unchallenged control group. Significant differences between the groups are indicated with different letters (P<0.05).

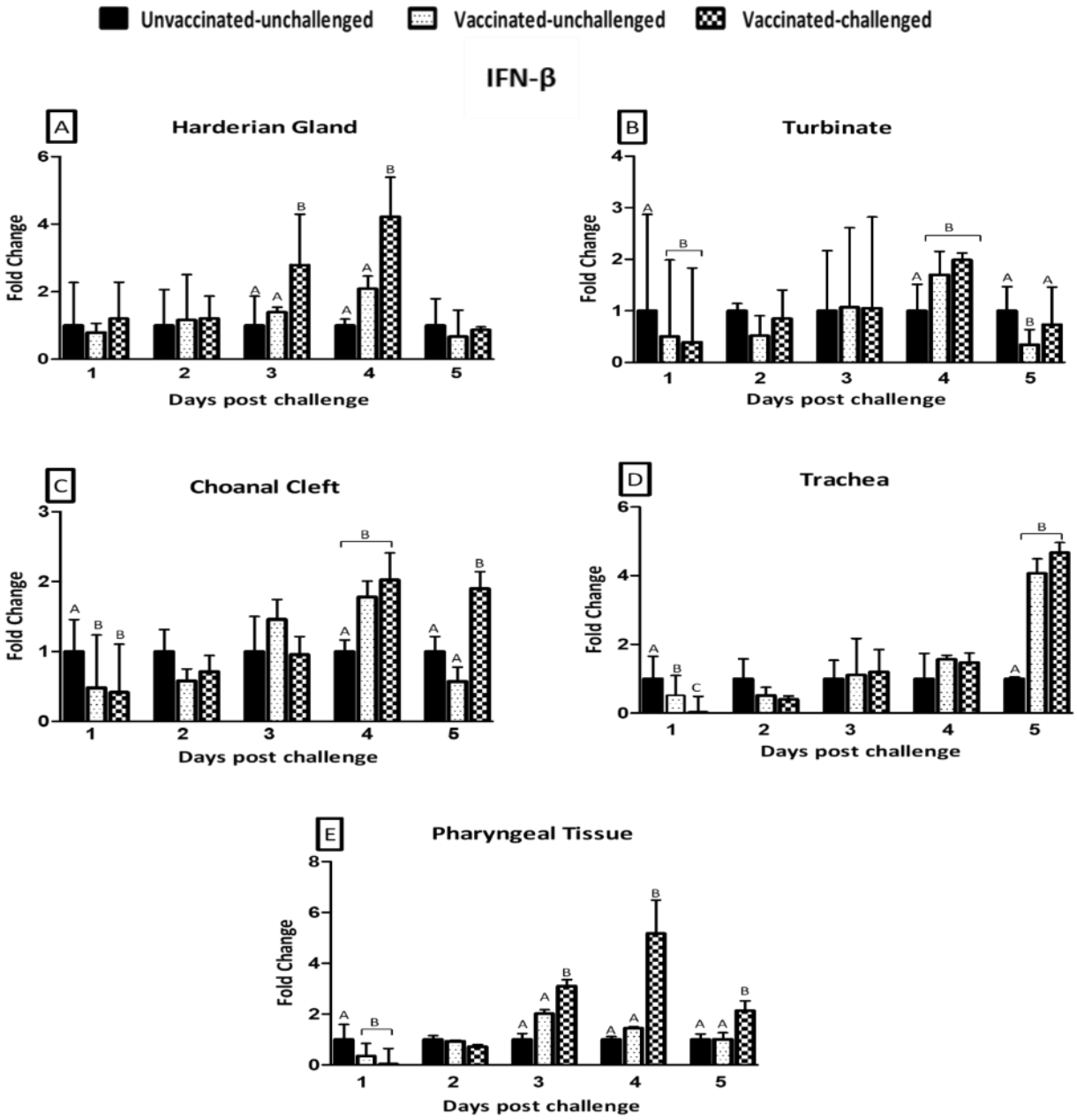

Figure 4.11. Relative mRNA expression of IFN-β (A-E) in the Harderian gland, turbinate, choanal cleft, trachea and pharyngeal tissue sample of chickens. Data represents the mean (n=5), with error bars indicating standard errors, and are expressed as fold change relative to the unchallenged control group. Significant differences between the groups are indicated with different letters ($P<0.05$).

4.3.10 Pro-inflammatory cytokine (IL-6) regulation

Harderian gland

There was a significant down-regulation of IL-6 mRNA expression at 1 dpc in the vaccinated-unchallenged group, and 4 and 5 dpc in the vaccinated-challenge group when compared with the control group (Figure 4.12 A).

Choanal cleft

There were only significant differences in IL-6 mRNA expression at 1 and 5 dpc in the vaccinated-challenged group in comparison to the control and vaccinated-unchallenged groups. (Figure 4.12 C).

Turbinate

There was significant up-regulation in the mRNA expression of IL-6 at 1 and 5 dpc in the vaccinated-challenged groups, whereas the vaccinated-unchallenged group was significantly down-regulated at 3 dpc compared to the control group (Figure 4.12 B).

Trachea

There was a significant up-regulation in the mRNA expression of IL-6 at 2 and 5 dpc in the vaccinated-unchallenged birds, and at 5 dpc in the vaccinated-challenged groups compared with the control group. Significant differences were noted between vaccinated and vaccinated-challenged groups at 2 and 5 dpc (Figure 4.12 D).

Pharyngeal tissue

There was a significant up-regulation in the mRNA expression of IL-6 at 2 and 5 dpc in the vaccinated-unchallenged group when compared to control group. Furthermore, there was a significant up-regulation in the mRNA expression of IL-6 at 5 dpc in the vaccinated-challenged group. There was a significant difference between vaccinated and vaccinated-challenged at 2 and 5 dpc (Figure 4.12 E).

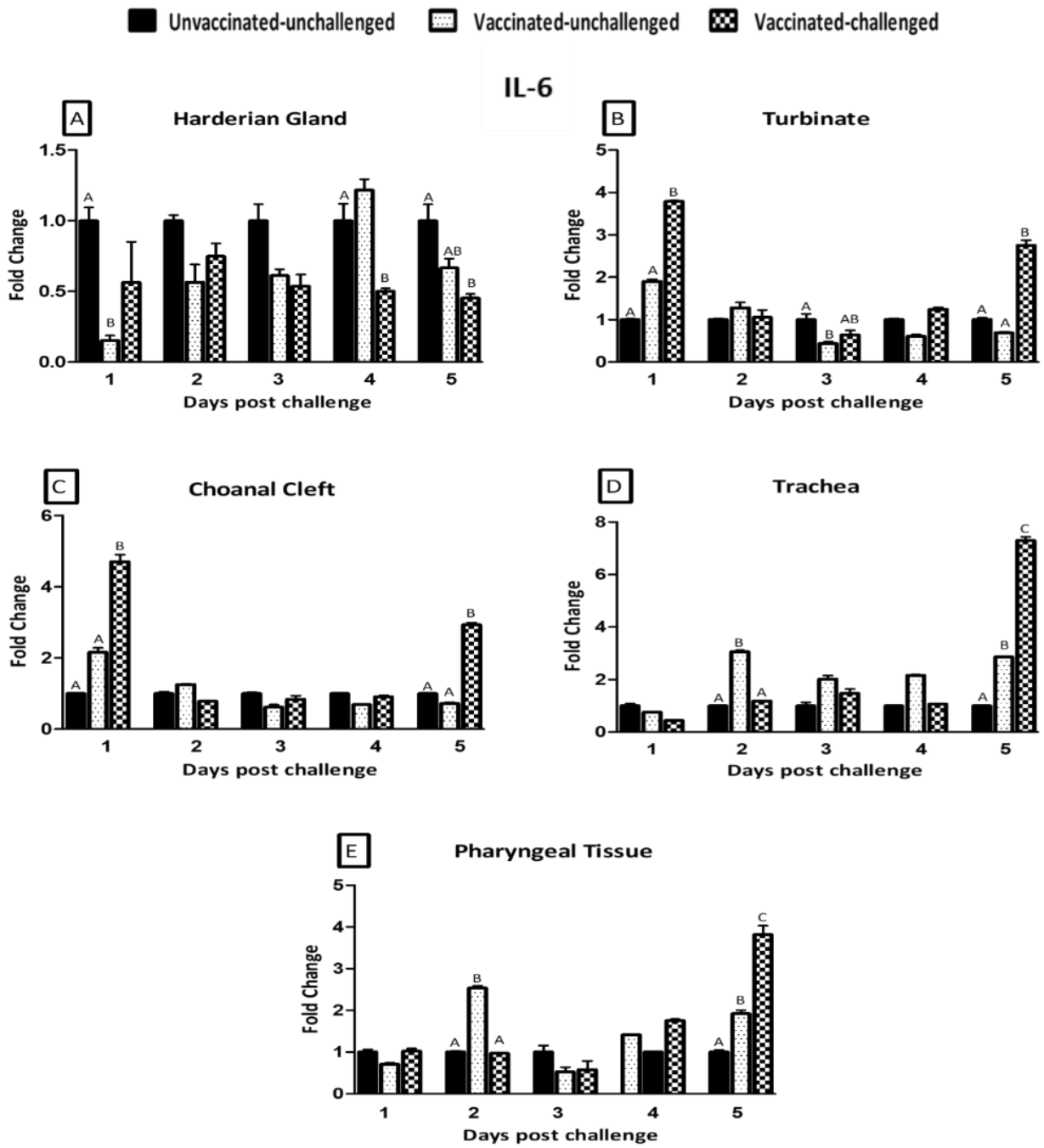

Figure 4.12. Relative mRNA expression of IL-6 (A-E) in the Harderian gland, turbinate, choanal cleft, pharyngeal tissue and trachea sample of chickens. Significant differences between the groups are indicated with different letters ($P<0.05$). Data represents the mean (n=5), with error bars indicating standard errors, and are expressed as fold change relative to the unchallenged control group. NO significant differences between the other groups indicated with letter AB.

4.4 Discussion

This is the first study to investigate the effects of classical virulent M41 on the expression of innate antiviral mediators in head-associated lymphoid and respiratory tissues in chicks previously vaccinated with combined live IBV (H120+793B). Although several pieces of research have demonstrated host-virus interactions using virulent and vaccine IBV strains (Okino *et al.* 2013; Yang *et al.* 2018), limited studies have investigated the broiler chicken's innate immune response in tissues other than the trachea. Vaccine regulatory bodies, such as the European Pharmacopeia, have been using the cilia-stopping test (Cook *et al.* 1999) as a main criterion of demonstrating protection against virulent IBVs. The cilia stopping test is very labour intensive and, more importantly, the readings are based on subjective scoring by individuals. In an attempt to introduce novel, quantitative, reliable and immune related protection indicators for IBV vaccine efficacy, this study reports on quantitative immune related assays as new IBV vaccine efficacy protection biomarkers. This can be used in association with the cilia-stopping test, tissue histopathology and challenge virus isolation or detection by molecular methods.

The cilia of vaccinated-challenged birds were protected against damage and showed high levels of activity, similar activity to the vaccinated-unchallenged and the unvaccinated-unchallenged chickens. In addition, the vaccinated-unchallenged birds also developed fewer tracheal lesions and had a lower viral load in the trachea. These results are in agreement with Ball *et al.* (2019) findings which showed high level of ciliary protection in day-old broiler chicks after combined (Massachusetts-H120 and 793B-CR88) vaccines following to M41 challenge. These results are also similar after a full dose of the attenuated H120 vaccine is applied on the first day of age followed by

IBV M41 challenge (Okino *et al.* 2013). These findings suggest that the combined vaccination protects birds against M41 to the same level as the individual challenge. It has been suggested that vaccine induced protection was primarily due to mucosal and cell-mediated immunity (Chhabra *et al.* 2015a).

Chickens from the vaccinated-unchallenged group showed mild tracheal damage. However, moderate histological lesions were observed in the trachea of vaccinated-challenged birds. Histological changes were marked in both groups at 1 dpc with both groups demonstrating high tracheal histopathology inflammation compared to the unvaccinated-unchallenged control. This inflammatory response was a result of the presence of intense infiltrate of mononuclear cells in the vaccinated-unchallenged birds, probably due to the transient and early migration of mononuclear cells to the primary site of IBV replication, developing from the participation of chemokines derived from immune-mediated T cells (Kotani *et al.* 2000b; Okino *et al.* 2013).

Day-old broiler chicks had anti-IBV ELISA antibody titre at 1 day old, which decreased to below the cut-off point at 21 dpv. These low levels of antibodies can result from partial neutralisation of the vaccine virus in the target tissues by the maternal antibodies present at that age. In addition to the high percentage of protection, there was a significantly higher antibody titre in the vaccinated-challenged group compared to the unvaccinated-challenged group. The observed increase in this group could mainly be attributed to re-stimulation of the humoral immune response to IBV challenge (Chhabra *et al.* 2015a; Mondal & Naqi 2001).

This study is the first to demonstrate that M41-challenge in vaccinated birds significantly raises the lachrymal IBV-specific IgA levels from 3 dpc onward, compared

to vaccinated-unchallenged birds. Also noted was a temporary loss of IgA levels at 1 dpc, which reflects possible neutralisation of pre-existing IgA due to prior vaccination. Previous studies have not reported such change, possibly due to sampling intervals omitting the early responses (Chhabra *et al.* 2015b; Da Silva *et al.* 2017; Okino *et al.* 2013). The increase in IgA was accompanied by a significant rise in IBV-specific IgY which might be primarily influenced by transduction or transudation (Ganapathy *et al.* 2005; Toro *et al.* 1993). Others studies have demonstrated that levels of IgA could be associated with the degree of protection against virulent IBVs (Chhabra *et al.* 2015b; Da Silva *et al.* 2017; Okino *et al.* 2013). This reflects that, in IBV vaccination-challenge studies, a quantitative assay of lachrymal IgA could be used as an early protection indicator.

Following the inoculation of the virus by the oculonasal route, it passes through tissues such as the turbinate, conjunctiva, infraorbital sinuses, HG, choanal cleft and pharyngeal tissues, eventually reaching the trachea. It was interesting to note that the trachea had significant viral load in the vaccinated-challenged chickens compared to the vaccinated-unchallenged at 1-4 dpc. It has not been reported before and though the reason for this is unclear, it is likely that the lymphoid tissues, including the lymphoid components in the turbinate (nasal-associated lymphoid tissues), were capable of eliminating the virus (Darbyshire *et al.* 1976; Darbyshire *et al.* 1978) compared to the tracheal epithelium. It appears that quantification of viral load in the HG, turbinate, choanal cleft or pharyngeal tissue may not be appropriate to demonstrate differences between the vaccinated-challenged and vaccinated-unchallenged. Instead, at least for the M41-challenge, the trachea is a more suitable tissue choice to demonstrate protection against challenge (Chhabra *et al.* 2015b; Okino

et al. 2013). Unlike the cilia-stopping test, viral load provides quantitative values for statistical comparison and should be considered in future IBV vaccination-challenge studies.

Findings in this study showed significant up-regulation of TLR3 and MDA5 in the HG, turbinate, choanal cleft and pharyngeal tissues of the vaccinated-challenged group compared to the vaccinated-unchallenged chickens. These quantifiable differences between challenged and unchallenged chickens could be used as a marker for assessing protection against IBV. Thus, this is time dependant on when the tissue sample is taken, therefore veterinarians and researchers would need to take onset of disease into consideration. In contrast, trachea samples from vaccinated-challenged birds showed a down-regulation of TLR3 at 2 dpc compared to vaccinated-unchallenged, accompanied by a prolonged expression of MDA5 compared to the other tissues. Notably, TLR3 was detected in the vaccinated-challenged group but not in the vaccinated-unchallenged (as also seen following challenge of unvaccinated chickens; Chapter 3), indicating that it is expressed soon after infection. Therefore, an up-regulation of both gene signatures can be used for the assessment of IBV vaccine efficacy. The innate immune response initiated by the multiple IBVs appeared to be mediated by TLR3 and MDA5, which activate intracellular pathways for the production of type one interferons and pro-inflammatory cytokines (Chhabra *et al.* 2015c; Kameka *et al.* 2014; Wang *et al.* 2006; Zhang *et al.* 2017).

Expression of IFN-α mRNA showed a significant increase at 1 dpc for the choanal cleft, turbinate and trachea in vaccinated-challenged birds compared to vaccinated-unchallenged, suggesting a useful parameter for measuring early host response.

Following a vaccination-challenge study, Yang *et al.* (2018) evaluated IFN-α and found similar results in tracheal tissue. IFN-β is the end product of the TLR3 and MDA5 pathways and has been reported in connection to protection against infection with different IBV strains, tissue tropism, histopathology and antiviral state (Chhabra *et al.* 2018; Okino *et al.* 2017). In this study, the pattern of mRNA expression of IFN-β demonstrated up-regulation in the HG at 3-4 dpc, whereas other tissues demonstrated both up- and down-regulation in both vaccinated groups, and it was thus difficult to establish a particular expression pattern. However, the differential patterns of expression make it unclear to definitively state a single factor as a biomarker. Rather it would be useful to use the overall pattern of expression to demonstrate infection.

Pro-inflammatory cytokines, including IL-6, are considered to be markers of inflammation and have been reported to be connected to tissue damage, increased viral load and increased tissue tropism (Asif *et al.* 2007; Chhabra *et al.* 2018). In the current study, significantly higher IL-6 was detected in the choanal cleft and turbinate at 1 and 5 dpc in the vaccinated-challenged group compared to the vaccinated-unchallenged group. Further studies will be needed to ascertain responses of other IBV challenge strains, as well as the inclusion of a wider range of gene signatures, inflammatory and proinflammatory cytokines and chemokines. Such comprehensive work would provide the most appropriate biomarker(s) for measurement of IBV vaccine efficacy.

In conclusion, the current experiment has demonstrated the kinetics of early immune responses in 21-day-old IBV-vaccinated broiler chickens following M41-challenge. In addition, it was shown that, other than the trachea, IBV actively replicates in

head- associated lymphoid (HG, choanal cleft and pharyngeal) and respiratory (turbinate and trachea) tissues, which appears to contribute in the infection and immunobiology of IBV. However, this is dependent on tissue type, with significant changes in TLR3, MDA5, IFN-α and IL-6 mRNA expression in the turbinate and trachea being the most notable. In a comparison between the vaccinated-unchallenged groups and vaccinated-challenged, significant differences were found in the following: i) mucosal immunity as reflected by lachrymal IBV-specific IgA, ii) gene signatures (TLR3 and MDA5), IFN-α and IL-6 in the choanal cleft, turbinate and trachea. While further studies are needed, data from the current study has, for the first time, provided a scientific foundation for the inclusion of quantitative parameters for measurement of protection in IBV vaccination-challenge studies, which could assist in transforming current vaccine efficacy testing protocols.

Chapter 5: Comparative protective immunity provided by infectious bronchitis vaccines administered by oculonasal, spray or gel routes in day-old commercial broiler chicks

Abstract

For commercial chickens, infectious bronchitis virus (IBV) vaccines can be administered by several different routes. To evaluate and cross-compare between vaccine application methods, three vaccination strategies were applied [gel, spray, and oculonasal (ON)] in day-old broiler chicks. Birds were then challenged at 21 days post vaccination (dpv) with M41, and protection status was evaluated at 5 days post challenge (dpc). Blood and lachrymal fluid were collected at 3, 7, 10, 14, and 21 dpv, and at 5 dpc for detection of systemic and local antibodies. Tissues (Harderian gland (HG), turbinates, choanal cleft, and trachea) were collected to quantify viral replication and host innate immune responses. All routes of vaccination resulted in an increase of specific IgA in the lachrymal fluid at 5 dpc compared to control group. For gel and ON vaccination methods, viral load in the turbinate, choanal cleft and trachea after 3-21 dpv, was higher than in the spray-vaccinated birds. For the oculonasal vaccinated-challenged birds (ON-ch), TLR3 mRNA expression was significantly up-regulated in all tissues at 1 dpc. Significant up-regulation of MDA5 and IL-6 mRNA was detected in the ON-ch, spray vaccinated-challenged (SP-ch) and gel vaccinated-challenged (Gel-ch) groups showed significant up-regulation in all tissues (except the HG-IL-6) compared to the control group at 1 dpc. Viral replication and host gene signatures are likely to be related to the difference in the tissue type and method of vaccine administration. It appears that the ON route of vaccination in broiler chicks provided superior protection after challenge with M41 due to tracheal protection based on the cilia stopping test and higher IgA levels. The ciliostasis data has also shown that birds vaccinated via gel were protected from virulent IBV M41, and this method could be an effective commercial vaccination approach against IBV.

5.1 Introduction

In chicken farming, vaccination against IBV is one of the core practices in avoiding losses due to virulent IBVs. The gold standard approach for vaccinating against IBV is oculonasal (ON) (de Wit 2013; Jackwood & de Wit 2013), but this is not practical for large numbers of birds. In the field, live vaccines are usually administered via drinking water (Gough & Alexander 1979; Ratanasethakul & Cumming 1983), coarse spray (Martin *et al.* 2007; Matthijs *et al.* 2005; Pensaert & Lambrechts 1994) or aerosols at day-old or within the first week of age (Jackwood & de Wit 2013). While these methods are popular due to convenience, there may be problems in achieving uniform application of the vaccine (Saif *et al.* 2003). For this reason, gel vaccination was recently suggested as a new method for IBV control and vaccine intake (Godoy *et al.* 2017; Jordan 2017). This vaccine delivery method is widely adopted in coccidiosis control strategies (Chapman *et al.* 2002). Negligible respiratory signs were detected in day-old broiler chicks during the 11 days of monitoring, even when combined strains (B-48 (Mass-like) and 1/96 (793B-like)) were administered by gel (Tucciarone *et al.* 2018). Gel droplets are rapidly ingested by preening (cleaning feathers with beak) optimises uptake due to the dye's appealing colour, and acts as a water supply to day-old chicks (Chapman 2000).

Different methods of administrating monovalent or bivalent vaccines at day-old has not been thoroughly investigated for IBV. After vaccination, innate local immunity is activated, followed by cellular immune responses. Different vaccine strains could induce a different protective level of innate immunity in addition to the humoral immune responses, and could be depending on the route of application (Rehmani 1996; Toro *et al.* 1997).

The innate immune plays a critical role in viral clearance when the virus is sensed through either TLR3 or MDA5 expression, which is considered the typical pathway for IBV to induce type 1 IFNs (Chhabra *et al.* 2015a). Thereafter, cells are recruited to the site of infection, which produce pro-inflammatory cytokines, such as IL-6 and IL-1β, which are regarded as inflammatory markers. This innate immune response differs according to the infective strain, target organs (Okino *et al.* 2017) and between vaccinated and challenged birds (Yang *et al.* 2018). For IBV, it has been claimed that higher levels of IL-6 and IL-1β are associated with increased lesions scores and increased viral load in infected trachea and kidney tissues (Okino *et al.* 2014). Recent studies have focused on studying the immune responses in the main organs, such as the trachea and the kidney (Chhabra *et al.* 2018; Okino *et al.* 2017; Smith *et al.* 2015), but neglect other tissues such as the Harderian gland (HG), turbinate and choanal cleft, which are in the head. The objective of this study is to cross-compare the protection and immunity induced by an attenuated IBV vaccines, which were administered by oculonasal, spray or gel methods in commercial broiler chicks.

5.2 Material and methods

5.2.1 Chicks

One hundred and sixty, day-old chicks were obtained from a commercial hatchery and kept until 26-day-old according to animal welfare guidelines, under strict biosecurity measures in an isolation unit at the University of Liverpool (Chapter 2.3). Chicks were reared on deep litter with antibiotic-free water and feed provided *ad libitum* (Chapter 2.4).

5.2.2 IBV vaccines

Two commercial live IBV vaccines were used in this study, Massachusetts serotype (H120) and 793B serotype (CR88). Vaccines were stored at 4 °C until required, and then dissolved in sterile distilled water (SDW) according to the manufacturer's instructions (Boehringer Ingelheim Animal Health Limited, UK). For combined vaccination, one vial of each live vaccine was mixed after preparation and retained on crushed ice. For the oculonasal route, each chick received 100 µl of vaccine or SDW via the ocular (50 µl) and nasal (50 µl) route according to the experimental design.

The gel vaccination was reconstituted with a gel pack in SDW according to the manufacturer's instructions (Animal Science Products Incorporated, USA) and administered to the group using a 100 µl pipette in a manner to simulate hatchery administration. After the vaccination, the chicks were left in the boxes for 30 mins allowing the vaccine ingestion by preening optimises uptake due to the dye's appealing colour. After the vaccination procedures, birds were individually checked for vaccine ingestion by tongue examination and dye presence evaluation and kept apart from each other to avoid vaccine contaminations.

For the spray vaccination, chicks were vaccinated using the standard protocol administered by the commercial hatchery with a droplet size of at least 100-150 microns. This was done using automated hatchery methods, which include either the use of a spray cabinet that is triggered every time a box of chicks is placed inside, or a spray vaccinator mounted over the conveyor line for chick boxes. Chicks were left in boxes for at least 20 mins after spraying, to optimise the effect of preening.

5.2.3 IBV challenge virus

A virulent strain of IBV M41 was used as the challenge virus. This virus was propagated and titrated in 10 days old specific-pathogenic-free (SPF) embryonated chicken eggs (ECE) (Chapter2.1). The eggs were inoculated via the allantoic cavity (Bijlenga *et al.* 2004). Virus rich allantoic fluid was collected and titrated ($10^{5.75}$ CD_{50}/bird) in chicken tracheal organ cultures (TOCs) (Chapter 2.2), and was confirmed to be free from other viruses, bacterial or fungal contaminants (Chapter 2.6).

5.2.4 Experimental study

One hundred and sixty, day-old chicks were divided into four initial groups (n=40) (Table 5.1); (A1) vaccinated by gel; (B1) vaccinated by spray; (C1) vaccinated by oculonasal; and (D1) unvaccinated. Chicks in the vaccinated groups received 0.1 ml of the combined Mass and 793B vaccine, via gel and ON routes. For spray, chicks were vaccinated with the combined vaccine using the standard protocol administered by the commercial hatchery. Birds in unvaccinated group were sham-inoculated with 0.1 ml of chilled vaccine-free SDW via ON route. At 21 day-post vaccination (dpv), birds were further divided into four groups (n=15) (Table 5.1); (A2) vaccinated by gel and challenge (Gel-ch); (B2) vaccinated by spray and challenge (SP-ch); (C2) vaccinated by oculonasal and challenge (ON-ch); and (D2) unvaccinated-challenged. Chickens in groups A2, B2, C2 and D2 were challenged with 0.1ml of M41 ($10^{5.75}$ CD_{50}/bird) by the oculonasal route. While chickens in the A1, B1, C1, and D1 groups, were mock challenged with 0.1ml of virus-free allantoic fluid by the same route. Birds were observed and inspected daily for clinical signs. Whole blood, oropharyngeal (OP) and cloacal swabs (CL) were collected from 10 chickens in each group at 3, 7, 10, 14 and 21 days post vaccination (dpv). Post challenge, at 1, 3 and 5 days post challenge (dpc) swabs were taken for virus

detection by RT-PCR, and serum collected for systemic antibodies detection. Lachrymal fluid was also collected from five chicks at 21 dpv and 5 dpc for anti-IBV antibody detection by monoclonal ELISA. Three chicks were humanely euthanized at 3, 7, 10, 14, and 21 dpv, and turbinate, choanal cleft and trachea tissues were collected for determination of viral load by quantitative real-time RT-PCR (qRT-PCR). Ciliostasis testing was conducted on tracheal rings at 5 dpc using a light microscope to confirm protection status. The Harderian gland (HG), turbinate, choanal cleft and trachea were collected at 1, 3, and 5 dpc from three birds for viral load quantification, and measurement of host gene response by qRT-PCR.

Table 5.1. Study design showing groups and vaccine method. At 21 days of age, chicks from each group were challenged with a virulent IBV M41 or unchallenged with virus-free allantoic fluid via ON route.

Group	Vaccination Method	Challenge Virus
A1	Mass + 793B by gel	Unchallenged
A2		M41
B1	Mass + 793B by spray	Unchallenged
B2		M41
C1	Mass + 793B by oculonasal	Unchallenged
C2		M41
D1	Unvaccinated	Unchallenged
D2		M41

5.2.5 Ciliostasis test

At 5 dpc, tracheas were extracted from five birds during necropsy and processed for ciliary protection scoring as previously described (Awad *et al.* 2016c; Cook *et al.* 1999; de Wit *et al.* 2011b). According to the European Pharmacopoeia (PhEur), individual birds yielding 50% or more tracheal explants with ciliary activity were considered to have been protected by vaccination against the challenge virus (Council of Europe 2007).

5.2.6 Swab samples

Ten OP and CL swabs were taken from each group at 3, 7, 10, 14 and 21 dpv, and 1, 3 and 5 dpc. Swabs from the same group were pooled, dipped into 3 ml of TOC medium and stored at -70 °C until required (Chapter 2.8.1).

5.2.7 Molecular detection of IBV genome

5.2.7.1 Extraction of RNA from swabs

RNA was extracted from OP and CL swabs using QIAamp viral RNA mini kit (Qiagen, UK) following the manufacturer's instructions (Chapter 2.12.2).

5.2.7.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

Following extraction, RT-PCR was conducted on swabs to detect a partial IBV S1 sequence (393bp) as previously described (Cavanagh *et al.* 1999; Ganapathy *et al.* 2015) (Chapter 2.14).

5.2.7.3 Extraction of RNA from tissue

Total RNA was extracted from all collected tissues using the RNeasy Plus Mini Kit (Qiagen, UK) according to the manufacturer's instructions (Chapter 2.13).

5.2.8 Real-time RT-PCR

Viral load was quantified from RNA extracted from the HG, choanal cleft, turbinate, trachea and swabs (Ball *et al.* 2016b; Chhabra *et al.* 2018; Jones *et al.* 2011a) (Chapter 2.20).

5.2.9 Host gene expression analysis

Host gene expression analysis was carried out for samples collected at 1, 3 and 5 dpc. Extracted RNA was converted to cDNA, and qPCR of cDNA samples was performed in triplicate using LightCycler 480 SYBR Green I Master mix and primers (Chapter 2.21, Table 2.5). Data were normalised against 18S ribosomal RNA expression using a relative standard curve method (Chhabra *et al.* 2018; Kuchipudi *et al.* 2012; Okino *et al.* 2013; Rajesh *et al.* 2016) and presented as fold change difference between virus- versus unvaccinated-unchallenged groups.

5.2.10 Serum for humoral antibody detection

Blood samples from the brachial vein were obtained from ten birds of each group at 3, 7, 10, 14, and 21 dpv and placed in labelled 5 ml bijous without coagulant overnight at room temperature. Serum was stored at -20 °C until further use (Chapter 2.8.2).

5.2.11 Lachrymal fluid for local antibodies detection

Lachrymal fluids were collected by pipette from all groups at 0 and 5 dpc using sodium chloride and immediately centrifuged at 3000 x g for 5 mins, and the supernatant was stored at -20 °C as previously described (Ganapathy *et al.* 2005) (Chapter 2.8.3).

5.2.12 Partial purification of IBV antigen and quantification using the Bradford Assay

IBV M41 was propagated in 10 days old chicken embryo eggs. The partial purification was carried out by adding 25 ml of allantoic fluid and centrifuging at 1,150 × g, 4 °C for 10 mins. The pellets were washed, re-suspended in phosphate-buffered saline (PBS)

and filtered through 0.22 µm filter. The virus suspensions were overlaid onto 30% sucrose and ultra-centrifuged at 102,400 × g, 4 °C for 4 h. Pellets were stored at -70 °C until use (Ganapathy *et al.* 2005; Keep *et al.* 2015) (chapter 2.16). The total concentration of the purified protein was quantified by using 5x Bradford assay (Chapter 2.17).

5.2.13 Serology

5.2.13.1 ELISA detection of IBV antibodies

A commercial IBV ELISA kit (IDEXX, Westbrook, Maine, USA) was used to determine anti-IBV antibodies for all groups according to the manufacturer's guidelines, with a positive ELISA titre cut-off determined to be 396 (Chapter 2.18.1).

5.2.13.2 Measurement of local antibodies against IBV

Lachrymal fluid for IBV-specific IgA and IgY at 5 dpc were assayed using an indirect ELISA (Dhinakar Raj & Jones 1996c; Ganapathy *et al.* 2005; Mockett & Cook 1986) (Chapter 2.18.2).

5.2.14 Statistical analysis

Data was analysed using the student's t-test or a one-way analysis of variance (ANOVA), followed by the post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00. Differences between groups were considered significant at $P<0.05$ (Chapter 2.22).

5.3 Results

5.3.1 Clinical signs and gross lesions

Following vaccination, mild clinical respiratory signs, such as head shaking, tracheal râles, and snicks appeared at 6 dpv and subsided by 19 dpv in the group vaccinated by oculonasal and gel routes. Similar signs were noticed in chickens vaccinated by spray,

which started to appear at 10 dpv, which was followed by a reduction of signs at 21 dpv.

Following challenge, clinical signs consisting of mild head shaking, eye scratching and snicks were observed at 1-2 dpc in the oculonasal-challenged (ON-ch) and spray-challenged (SP-ch) groups, and at 1-5 dpc for the gel-challenged (Gel-ch) group. No gross lesions were found in any of the vaccinated-unchallenged (Gel-unch), (SP-unch) and (ON-unch), and vaccinated-challenged groups.

5.3.2 Ciliostasis

Ciliary activity in the trachea was measured at 5 dpc. All the vaccinated-unchallenged groups and vaccinated-challenged groups (Gel-ch, SP-ch, and ON-ch) showed a minimum average ciliary protection of approximately 80-90% whereas that of the unvaccinated-unchallenged (Unv-unch) control was > 95% (Figure 5.1).

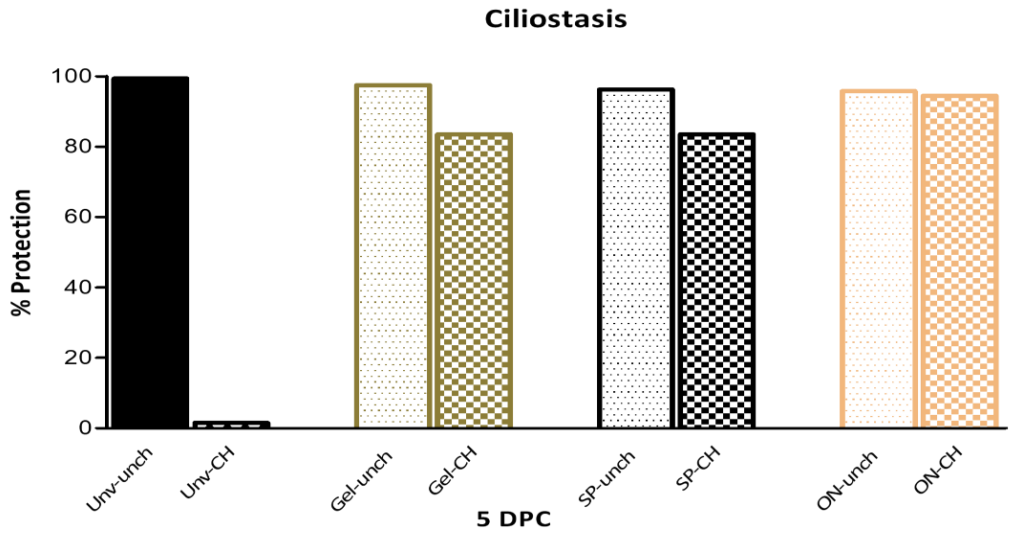

Figure 5.1. Ciliary protection against the IBV M41 challenge virus according to the European Pharmacopoeia's reference standards, individual birds yielding 50% or more tracheal explants with ciliary activity were considered to have been protected by the vaccine.

5.3.3 Detection of vaccine viruses by RT-PCR

No virus was detected in the unvaccinated-unchallenged group throughout the study. For IBV-vaccinated groups, IBV was detected in OP swabs in all vaccinated groups from 3 to 21 dpv. For CL swabs, IBV was also detected in all vaccinated groups from 3 to 21 dpv in the gel and the ON groups. However, in the spray-vaccinated group, IBV was only detected after 7 dpv (Table 5.2).

Table 5.2. Detection of the vaccine virus of (H120+CR88) by RT-PCR in the OP and CL swabs at 3, 7, 10, 14, and 21 dpv. PCR was carried out on 10 birds.

DPV	Oropharyngeal (Number of birds positive out of 10)			Cloacal (Number of birds positive out of 10)		
	Gel	Spray	Oculonasal	Gel	Spray	Oculonasal
3	6	7	10	4	0	1
7	7	8	10	5	1	6
10	10	9	10	8	3	9
14	10	10	10	8	9	9
21	10	10	10	10	9	10

5.3.4 Systemic anti-IBV-specific antibody titre using ELISA

High levels of IBV maternal-derived antibody (MDA) were detected from serum in all birds at day-old (2594±326). At 14 and 21 dpv, antibody titres had declined significantly to below the cut-off point (396) in the unvaccinated-unchallenged control group. In the spray and ON-vaccinated groups, antibody titres were above the positive cut-off point at 3 dpv (1510±274 and 1803±415 respectively) and 7 dpv (1000±227 and 992±116 respectively), and then decreased significantly by 10, 14 and 21 dpv in the spray (205±46, 109±80 and 121±47) and ON-vaccinated birds (24±18, 23±22 and 111±41). Positive antibody titres were detected in the gel-vaccinated birds at 3, 7 and 10 dpv

(2107±492, 562±115 and 433±96), titres then significantly declined at 14 and 21 dpv (159±115 and 85±50). A significant higher antibody titre was seen in the gel-vaccinated group compared with the ON-vaccinated and control group at 10 dpv (Figure 5.2).

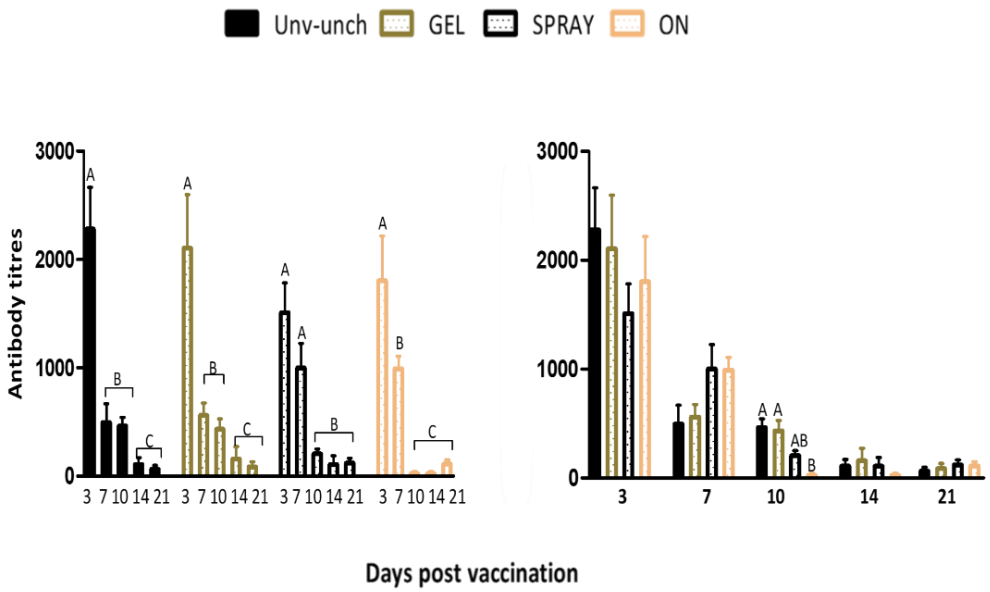

Figure 5.2. Infectious bronchitis virus (IBV) antibody titres (IgY) of the unvaccinated-unchallenged control and different vaccinated groups. Data are expressed as mean values ± SEM (n=10/group) at 3, 7, 10, 14 and 21 day-post vaccination. Significant differences between the groups were detected by one-way ANOVA test. Different letters indicate significant differences within groups. Cut-off point (396).

5.3.5. Mucosal anti-IBV- specific lachrymal antibodies (IgA) and (IgY) using indirect ELISA

5.3.5.1 Specific anti-IBV IgA

The titres of anti-IBV IgA in all vaccinated-unchallenged groups (gel, spray and ON) were detected at 21 dpv (0 dpc) (1.232±0.168, 1.513±0.193 and 1.719±0.183 respectively). There were no statistical differences between these groups. By 5 dpc (26 days of age), the titres declined to 0.924±0.170, 0.994±0.144 and 1.132±0.165

respectively but remained to have significantly higher titres compared to the unvaccinated-unchallenged group. The level of IgA at 5 dpc in all vaccinated-challenged chickens was significantly higher than the unvaccinated-unchallenged group (1.350±0.187, 1.459±0.095 and 1.700±0.115 respectively). There was a significantly high IgA titre in spray and ON vaccinated-challenged groups at 5 dpc compared to the vaccinated-unchallenged group (Figure 5.3 A). No statistical difference was observed between vaccinated-unchallenged and vaccinated-challenged groups at 5 dpc.

5.3.5.2 Specific anti-IBV IgY

At 21 dpv, IgY was detected in all vaccinated-unchallenged groups (gel, spray and ON) (0.253±0.043, 0.287±0.006 and 0.299±0.031 respectively), followed by a sharp reduction in levels at 5 dpc (26 dpv) in birds vaccinated by gel or spray (0.021±0.027 and 0.114±0.026 respectively). The IgY titre in chickens immunised by the ON route remained significantly higher measurable (0.179±0.068) compared to the unvaccinated-unchallenged group. The IgY level in the different routes of vaccinated-challenged (Gel-ch, SP-ch and ON-ch) were significantly higher at 5 dpc (0.292±0.099, 0.352±0.201 and 0.334±0.031 respectively) compared to the vaccinated-unchallenged groups (Figure 5.3 B). No statistical difference was noted between vaccinated groups at 5 dpc. Data for unvaccinated-challenged groups have presented in Chapter 3.

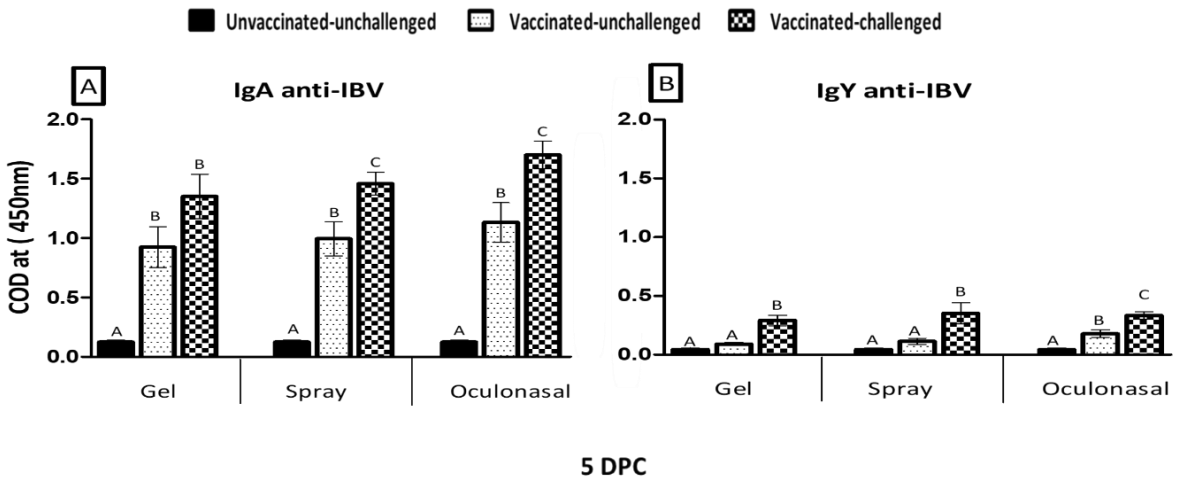

Figure 5.3. Measurement of IBV-specific IgA and IgY titre using indirect ELISA from the lachrymal fluid. Mean IgA and IgY from lachrymal fluids collected at 5 dpc (n=5), data expressed as mean of corrected optical density (COD). Significant differences are indicated with different letters.

5.3.6 Viral RNA quantification

5.3.6.1 Swabs

Viral RNA copies in oropharyngeal and cloacal swabs was measured using qRT-PCR (Table 5.3; Figure 5.4). All swab samples collected from vaccinated groups were positive on all sampling days, with the control group negative throughout the study.

Post vaccination: Viral RNA load in OP swabs peaked at 21 dpv in birds vaccinated by gel and spray (2.95 log REU and 2.81 log REU respectively), while the ON-vaccinated birds peaked earlier at 7 dpv (2.37 log REU). Viral RNA load in the CL swabs was positive from 3-21 dpv in the gel- and ON-vaccinated groups, and from 7-21 dpv in the spray-vaccinated birds. Virus presence peaked at 14 and 21 dpv in the birds vaccinated by gel and spray respectively (2.04 log REU and 3.34 log REU), while the ON-vaccinated birds peaked earlier at 10 dpv (2.81 log REU).

Post challenge: Viral RNA load in the OP swabs from the gel-unch and the ON-unch birds peaked at 5 dpc (2.21 log REU and 2.69 log REU respectively), while the SP-unch peaked earlier at 3dpc (2.10 log REU). For the challenged groups, the gel-ch and SP-ch groups peaked at 1 dpc (2.67 log REU and 2.82 log REU respectively), while the ON-ch peaked later at 3 dpc (2.22 log REU). The viral load at 5 dpc was lower in the SP-ch and ON-ch groups compared to the vaccinated-unchallenged groups (Figure 5.4 A and C). The viral RNA load in the CL swabs from the gel-unch, spray-unch and ON-unch birds peaked at 3 dpc (3.09 log REU, 2.68 log REU and 2.21 log REU respectively). Whereas, viral RNA copies in all vaccinated-challenged groups peaked at 1 dpc (3.81 log REU, 2.72 log REU and 3.05 log REU respectively). The viral load in the gel and spray vaccinated-unchallenged groups was higher than the respective vaccinated-challenged groups at 3 and 5 dpc, but this was not true for the ON-vaccinated groups (Figure 5.4 B and D).

Table 5.3. Detection of the vaccine virus of (H120+CR88) by qPCR in the OP and CL swabs at 3, 7, 10, 14, and 21 dpv. PCR was carried out on pooled samples, and data is given as log REU.

DPV	Oropharyngeal log REU			Cloacal log REU		
	Gel	**Spray**	**Oculonasal**	**Gel**	**Spray**	**Oculonasal**
3	2.65	2.07	2.22	0.31	0.00	1.47
7	2.69	2.13	2.38	0.85	0.11	1.77
10	2.80	2.32	2.06	1.51	0.44	2.82
14	2.82	2.81	1.99	1.95	3.34	2.46
21	2.96	2.81	1.73	2.04	2.72	2.53

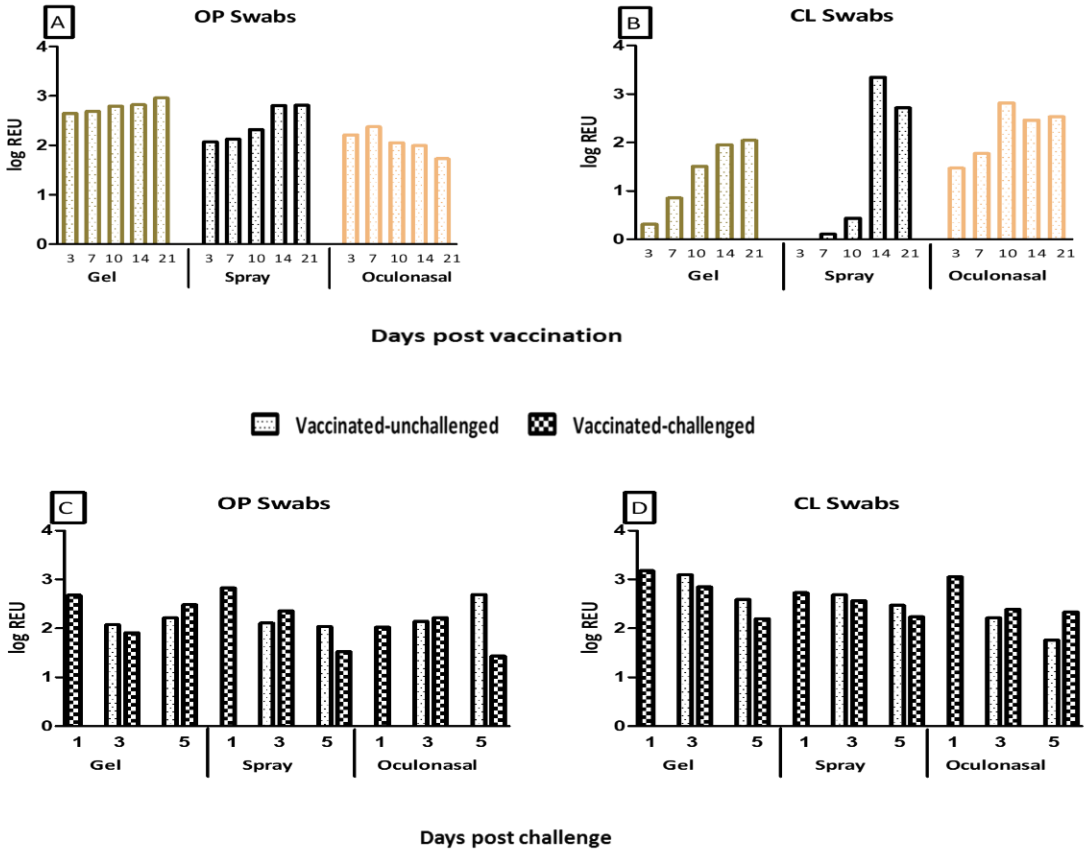

Figure 5.4. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA. (A and B) IBV loads at 3, 7, 10, 14 and 21 dpv in pooled OP and CL swabs. Chickens (n=10) were vaccinated with combined IBV strains (H120+CR88). (C and D) IBV loads at 1, 3 and 5 dpc in the pooled OP and CL swabs respectively. Vaccinated birds were challenged with IBV M41 at a dose of $10^{5.75}$ CD_{50}/bird. Data from the vaccinated-unchallenged groups have not processed at 1 dpc.

5.3.6.2 Tissues

5.3.6.2.1 Post vaccination

Tissue samples collected from vaccinated chickens from all groups were positive on all days.

The viral RNA loads in the ON-vaccinated birds, the viral RNA loads peaked earlier at 3 dpv (3.50 log REU). While in the in the turbinate of gel-vaccinated birds peaked at 10 dpv, and at 14 dpv in the spray-vaccinated birds (3.77 log REU and 2.88 log REU

respectively). The viral load in turbinate of the spray-vaccinated group was significantly lower compared with gel and the ON-vaccinated birds at 10 dpv (Figure 5.5 A1 and A2).

For the choanal cleft, viral RNA loads in peaked at 3 dpv in spray and ON routes (3.22 log REU and 2.72 log REU respectively) and at 7 dpv (3.95 log REU) in the gel-vaccinated birds. The viral titre in the choanal cleft was significantly higher in the gel-vaccinated group compared to other routes at 7 dpv (Figure 5.5 B1 and B2).

The viral load level in the trachea peaked earlier in chickens vaccinated by the ON route at 3 dpv (2.57 log REU) followed by a significant lower titre at 21 dpv than the previous days. The viral RNA loads in the trachea of gel-vaccinated bird peaked at 14 dpv (2.63 log REU) with significant high titres at 14 and 21 dpv compared to the previous sampling days, while in the spray-vaccinated birds, viral RNA loads peaked at 10 dpv (0.73 log REU) without any significant differences noted between sampling days. The viral titre in the trachea of spray-vaccinated birds was significantly lower compared to that of other routes at all sampling points. In addition, the viral copy in the trachea of gel-vaccinated birds was significantly high compared to the ON group at 14 and 21 dpv (Figure 5.5 C1 and C2).

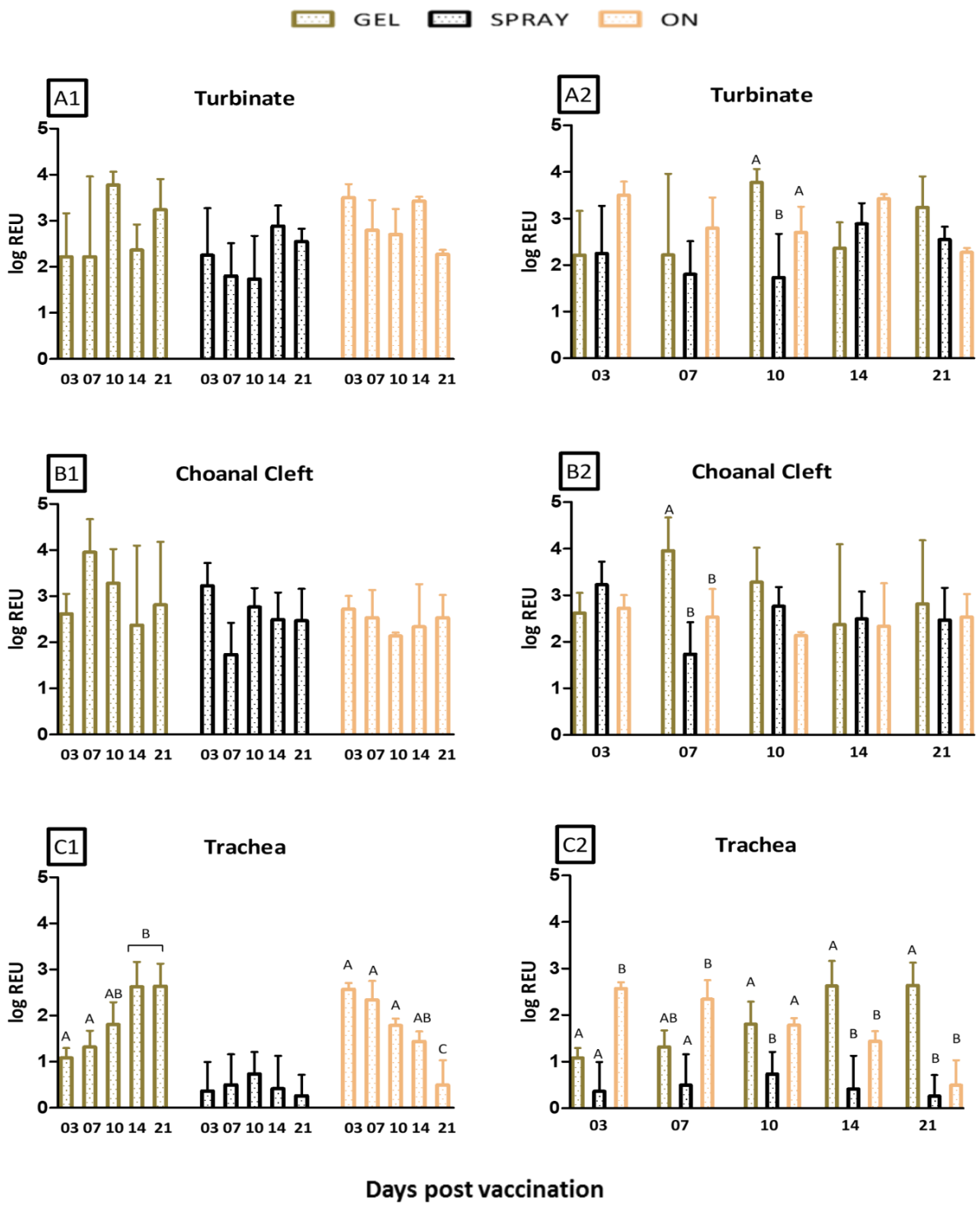

Figure 5.5. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA in the (A1) turbinate, (B1) choanal cleft and (C1) trachea samples of chicken at 3, 7, 10, 14 and 21 dpv. (A2-C2) Comparison between the different routes on the same tissue and day. Chickens were vaccinated with combined IBV strains (H120+CR88). Significant ($P<0.05$) differences between the groups indicated with different letters (n=3). Significant differences were determined using one-way ANOVA.

5.3.6.2.2 Following M41-challenge

Tissue samples collected from vaccinated-unchallenged and vaccinated-challenged chickens from all groups were positive on all days.

Viral copy numbers peaked at 3 dpc in the HG in the vaccinated-unchallenged birds. The viral titre in the HG was significantly higher in the respective vaccinated-unchallenged groups compared to the vaccinated-challenged group at 5 dpc (gel-vaccinated) and 3 dpc (spray- and the ON-vaccinated). (Figure 5.6 A1 and A2).

In the turbinate, the viral RNA peaked at 5 dpc in all vaccinated-unchallenged groups. The turbinate viral load was significantly higher in the vaccinated-unchallenged compared to the gel- and spray-vaccinated-challenged groups at 5 dpc. However, the viral copy in the turbinate at 3 dpc was significantly higher in the ON vaccinated-challenged compared to the vaccinated-unchallenged groups. A comparison between the methods demonstrates that the viral titre was significantly higher in the gel- and spray-vaccinated-unchallenged groups compared to the ON route at 3 dpc, while in the vaccinated-challenged groups, the significantly high titre was detected at 3 dpc in the ON route compared to the spray (Figure 5.6 B1 and B2).

In the choanal cleft, viral RNA in the vaccinated-unchallenged groups peaked at 3 dpc, with the exception of the ON-vaccinated group, which peaked later at 5 dpc. Similar to the turbinate, the viral replication titre in the choanal cleft was significantly higher at 3 dpc in the vaccinated-challenged compared to the vaccinated-unchallenged groups in the ON-vaccinated group. (Figure 5.6 C1 and C2).

With regard to the trachea, viral RNA levels in vaccinated-unchallenged chickens peaked at 3 dpc in gel and ON, and at 5 dpc in spray-vaccinated groups. The viral load

of RNA in the trachea was significantly higher at 3 dpc in gel-vaccinated birds, and at 3 and 5 dpc in the spray-vaccinated birds compared to the vaccinated-challenged birds. However, the tracheal viral copy at 3 dpc was significantly higher in the vaccinated-challenged compared to the vaccinated-unchallenged groups in the ON-vaccinated group. A comparison between the different methods shows that the viral titre was significantly higher in the SP-unch at 5 dpc compared to other routes, while in the vaccinated-challenged groups, a significant high titre was seen in the ON route at 3 and 5 dpc compared to the other methods, and only at 1 dpc compared to SP-ch group (Figure 5.6 D1 and D2).

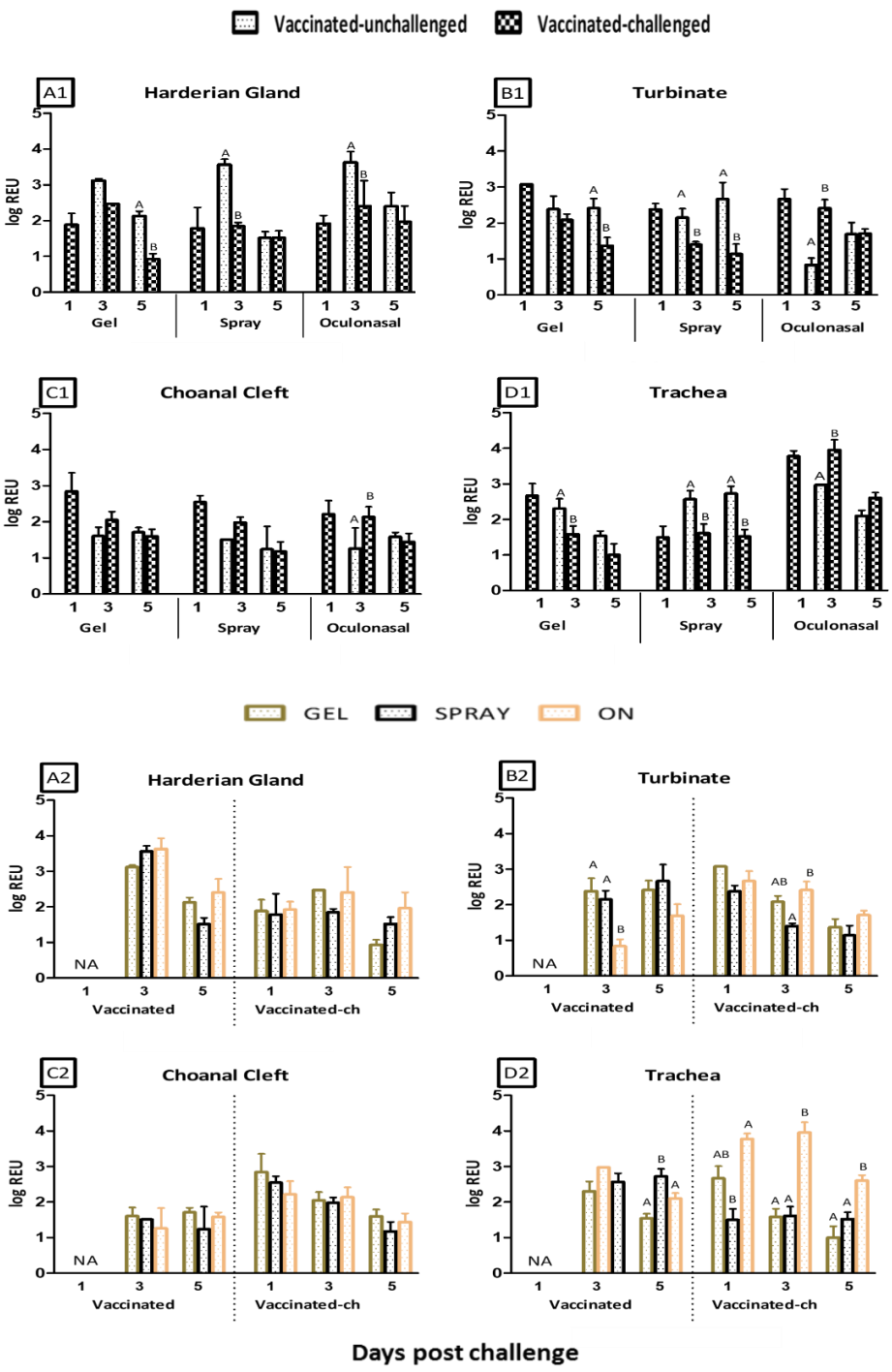

Figure 5.6. Quantification of viral RNA expressed as log relative equivalent units (REU) of RNA in (A1) Harderian gland, (B1) turbinate, (C1) choanal cleft and (D1) trachea (dpc). (A2-D2) Comparison between the different routes on the same tissue and day. Vaccinated birds were challenged with IBV M41 at a dose of $10^{5.75}$ CD_{50}/bird. Significant ($P<0.05$) differences between the groups (n=3) indicated with different letters. Significant differences were determined using one-way ANOVA. Data from the vaccinated-unchallenged groups have not been processed at 1 dpc and indicated with (NA) "not available".

5.3.7 Expression of TLR3 and MDA5 mRNA

Harderian gland (HG)

TLR3 mRNA expression in the HG was significantly down-regulated at 3 and 5 dpc in the groups vaccinated by gel and spray, and at 5 dpc in the ON-vaccinated group compared to the unvaccinated-unchallenged control group. In the vaccinated-challenged groups, there was significant down-regulation at 1-5 dpc in the Gel-ch and SP-ch groups, while in the ON-ch group, a significant up-regulation was present at 1 and 3 dpc compared to the control group. There was a significantly high difference in the TLR3 mRNA expression for the HG in the vaccinated-unchallenged group compared to vaccinated-challenged group at 5 dpc in the gel and spray-vaccinated groups, and at 3 and 5 dpc in the ON-ch group (Figure 5.7 A1). In comparison between all vaccination methods, a significant low expression was seen in the ON-unch birds at 5 dpc. However, in the vaccinated-challenged groups, TLR3 mRNA expression was significantly higher in the ON-ch group at all-time points (Figure 5.7 A2).

Expression of MDA5 in the HG showed significant up-regulation at 3 and 5 dpc in the groups vaccinated by gel and spray, and at 3 dpc in the ON-vaccinated birds compared to the control. In the vaccinated-challenged groups, significant up-regulation of the MDA5 was observed at 3 and 5 dpc in the Gel-ch group, and at all sampling days in both SP-ch and ON-ch groups compared to the control and vaccinated-unchallenged groups at 3 and 5 dpc (Figure 5.8 A1). Spray-vaccinated birds showed a significantly higher level of MDA5 mRNA at 5 dpc compared to the other routes. Moreover, a significant high difference in the mRNA expression of MDA5 in HG was seen in the spray-vaccinated group at 5 dpc compared to others groups, and in the SP-ch and ON-ch groups at 1 dpc compared to Gel-ch (Figure 5.8 A2).

Choanal cleft

TLR3 mRNA expression in the choanal cleft was significantly down-regulated at 3 and 5 dpc followed by significant up-regulation at 5 dpc in the gel-vaccinated groups. In the groups vaccinated by spray and ON methods, results indicated a significant down-regulation at 3 and 5 dpc compared with control. In the Gel-ch and SP-ch groups, there was significant down-regulation at 3-5 dpc. There was only significant up-regulation at 1 dpc in the ON-ch group compared to the control group. In addition, a significant high difference was detected at 5 dpc in the gel-vaccinated compared with the vaccinated-challenged group (Figure 5.7 C1). In comparison between all vaccination methods, a significant high expression was only noted in the ON-ch group at all-time points (Figure 5.7 C2).

MDA5 mRNA expression in the choanal cleft was significantly down-regulated at 3 dpc, followed by a significant up-regulation at 5 dpc in both gel- and spray-vaccinated chickens compared to the control group. In the vaccinated-challenged groups, a significant up-regulation of MDA5 was observed at 1 and 5 dpc in the SP-ch group, and at all sampling days in the both Gel-ch and ON-ch groups compared to the control group. A significant high difference was seen in the ON-ch group compared to ON-vaccinated birds at 3 -5 dpc (Figure 5.8 C1). A significant high difference was detected in the mRNA expression of MDA5 at 1 dpc in the SP-ch group (Figure 5.8 C2).

Turbinate

Significant down-regulation was noted in the mRNA expression of TLR3 at 3 dpc in the gel-vaccinated group, and at 3-5 dpc in both groups vaccinated via spray and the ON routes. The significant up-regulation was noted only in gel-vaccinated birds at 5 dpc compared with the control. In the vaccinated-challenged groups, there was significant

down-regulation at all-time points in the Gel-ch and SP-ch groups. Conversely, this gene detected significantly up-regulated in the ON-ch birds at all-time points compared to the control. Significant high differences were observed in the gel-vaccinated compared with Gel-ch groups at 5 dpc, and in the ON-ch compared with ON-vaccinated birds at 3 and 5 dpc (Figure 5.7 B1). A significant high difference in the TLR3 mRNA expression presented in the gel-vaccinated bird at 5 dpc and in the ON-ch at all sampling days in the vaccinated-challenged groups (Figure 5.7 B2).

Expression of MDA5 mRNA in the turbinate was significantly up-regulated at 5 dpc in the gel-vaccinated group, at 3 and 5 dpc in the spray-vaccinated birds and at 3 dpc in the ON group compared with the control group. In the vaccinated-challenged groups, there was significant up-regulation of the MDA5 at 1 and 5 dpc in the Gel-ch and SP-ch groups, and at 1-3 dpc in the ON-ch group compared to the control group. There was significant high difference in the SP-ch compared with spray-vaccinated groups at 5 dpc (Figure 5.8 B1). A comparison between the different vaccine administrations showed a significant high difference in the gel-vaccinated bird at 5 dpc. In the vaccinated-challenged groups, the higher significant expression levels were seen in both Gel-ch and ON-ch compared to SP-ch at 1 dpc. However, MDA5 expression presented significantly higher in the SP-ch at 5 dpc (Figure 5.8 B2).

Trachea

Significant up-regulation was noted in the mRNA expression of TLR3 at 3 dpc, and at 3-5 dpc in the gel- and ON-vaccinated groups respectively. The significant down-regulation of this gene was observed at 3 dpc in birds vaccinated via spray method compared to the control. In the vaccinated-challenged groups, significant down-regulation was noted at all sampling days in the Gel-ch and SP-ch groups. The opposite

was true for the ON-ch when this gene significantly up-regulated at all-time points compared to the control. Significant high differences were observed in the gel-vaccinated compared to Gel-ch at 3 and 5 dpc, and in the ON-ch compared to ON-vaccinated groups at 3 dpc (Figure 5.7 D1). A significant high difference in the mRNA expression of TLR3 expression noted at 3 dpc in the gel and at 5 dpc in the ON-vaccinated groups, and at all sampling days in the ON-ch for the vaccinated-challenged groups (Figure 5.7 D2).

MDA5 mRNA expression in the trachea showed significant up-regulation at all sampling days in the all vaccinated birds, and in vaccinated-challenged groups compared to the control group. Significant high differences were detected between both vaccinated groups in the gel- and spray-vaccinated groups at 5 and 3 dpc respectively, and at 3 and 5 dpc in the ON-ch for the vaccinated-challenged group (Figure 5.8 D1). A significant high difference in the mRNA expression of MDA5 expression noted in the gel-vaccinated birds at 5 dpc. In the vaccinated-challenged groups, a significant high difference was present at 1 dpc in the ON-ch, and at 3 dpc in the both Gel-ch and the ON-ch (Figure 5.8 D2).

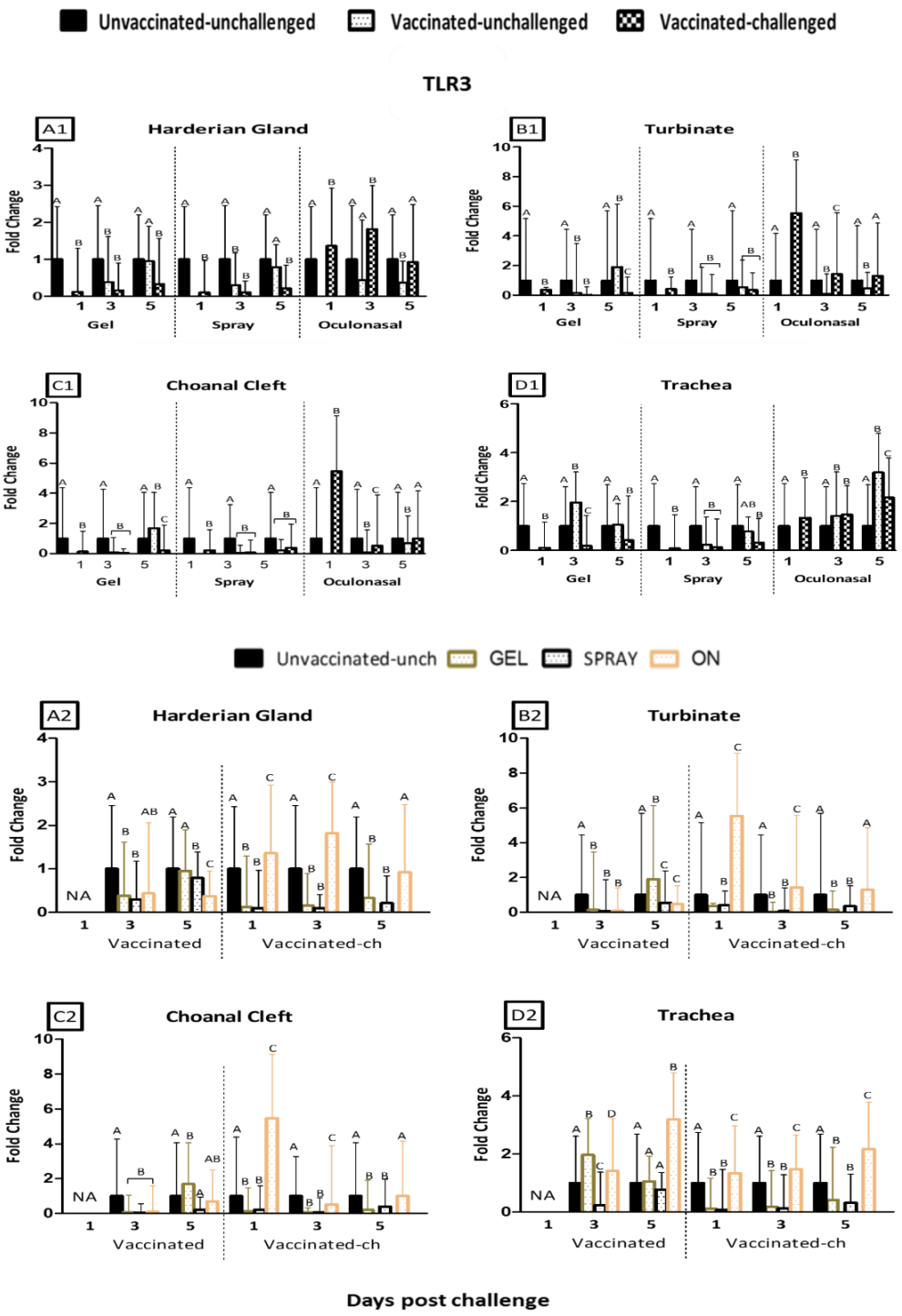

Figure 5.7. Relative mRNA expression of (A1-D1) TLR3 in the Harderian gland, turbinate, choanal cleft and trachea sample of chickens. (A2-D2) Comparison between the different routes on the same tissue and day. Significant differences between the groups indicated with different letters. Data represents the mean with error bars as standard error and are expressed as fold change relative to the unvaccinated-unchallenged control groups (n=3). Data from the vaccinated-unchallenged groups have not been processed at 1 dpc and indicated with (NA) "not available".

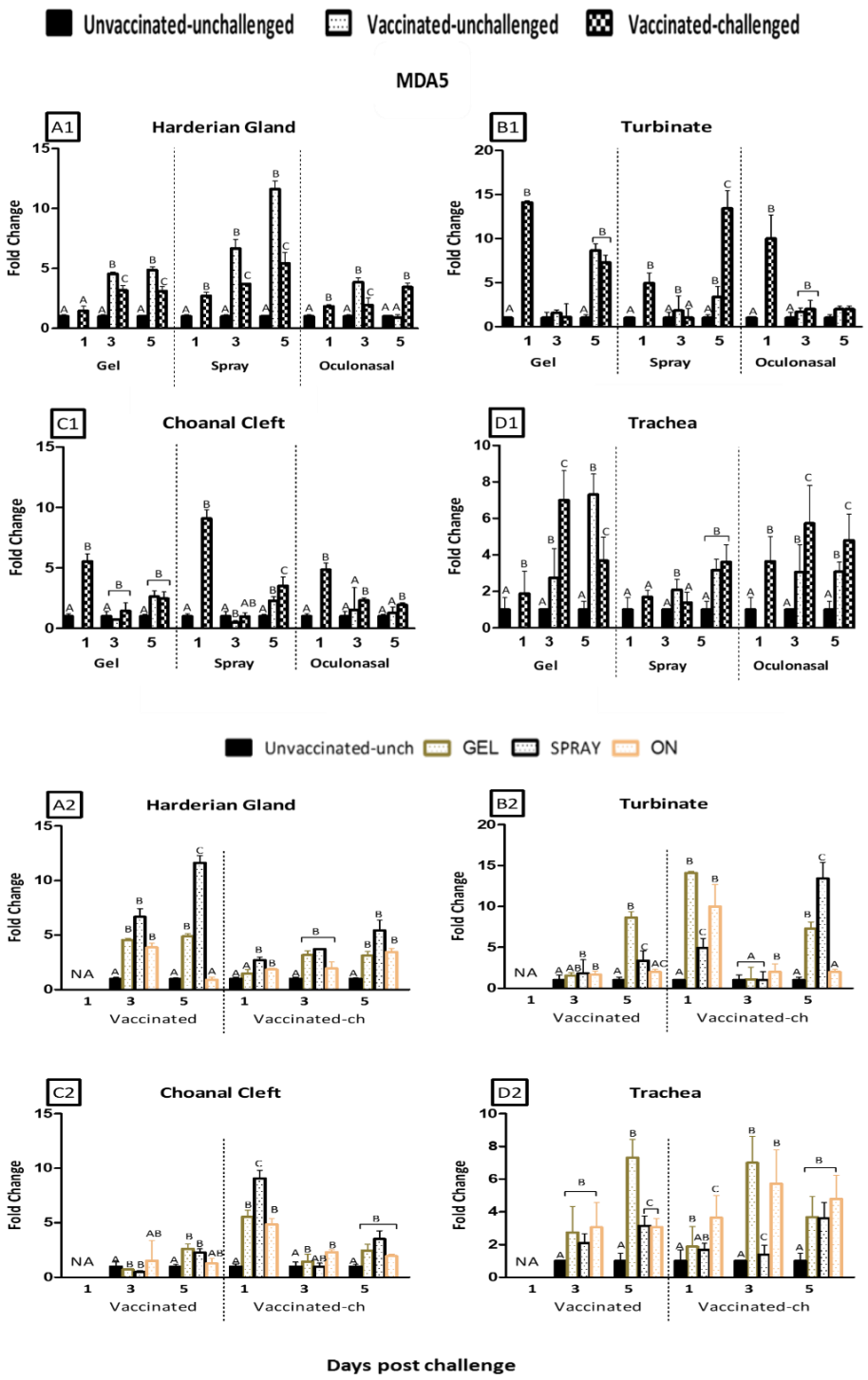

Figure 5.8. Relative mRNA expression of (A1-D1) MDA5 in the Harderian gland, turbinate, choanal cleft and trachea sample of chickens. (A2-D2) MDA5 presents a comparison between the different routes on the same tissue and day. Significant differences between the groups indicated with different letters. Data represents the mean with error bars as standard error and are expressed as fold change relative to the unvaccinated-unchallenged control group (n=3). Data from the vaccinated-unchallenged groups have not been processed at 1 dpc and indicated with (NA) "not available".

5.3.8 Expression of host IFN-α and IFN-β mRNA

Harderian gland (HG)

Significant down-regulation of IFN-α was seen on all sampling days in both vaccinated-unchallenged and vaccinated-challenged birds for all different vaccination routes compared to the control group. There was only a significant high difference was seen at 3 and 5 dpc in the vaccinated-unchallenged compared to vaccinated-challenged birds in the gel- and ON-vaccinated groups respectively (Figure 5.9 A1). The mRNA expression of IFN-α were significantly lower in spray-vaccinated birds at 3 dpc, and at 5 dpc in the gel-vaccinated group compared to the different routes of vaccine in the HG (Figure 5.9 A2).

There was significant down-regulation in IFN-β mRNA expression at 3 dpc in the spray-vaccinated group and in the vaccinated-challenged birds at all sampling days in both Gel-ch and SP-ch groups, while in the ON-ch birds, a significant down-regulation was observed at 5 dpc compared to the control group. The significant high difference was detected in the vaccinated-unchallenged compared to vaccinated-challenged birds at 5 dpc in the gel and the ON, and at 3 dpc in the spray-vaccinated groups respectively (Figure 5.10 A1). The mRNA expression of IFN-β was significantly higher in the ON-ch group when compared to other vaccination routes at 1 and 3 dpc (Figure 5.10 A2).

Choanal cleft

There was significant down-regulation in the mRNA expression of IFN-α in all birds vaccinated by different methods and also in the vaccinated-challenged groups of Gel-ch and SP-ch at 3 dpc. The significant up-regulation was observed in the ON-ch group at 1 dpc compared to the control group. There was a high significant difference in the ON-ch compared to ON-vaccinated birds at 3 dpc (Figure 5.9 C1). A significant high level

showed in the ON-vaccinated group at 3 dpc, and in the ON-ch group at 1 and 3 dpc (Figure 5.9 C2).

There was significant down-regulation of IFN-β mRNA expression in both vaccinated-unchallenged and vaccinated-challenged for all different vaccination routes at 3 dpc (Figure 5.10 C1).

Turbinate

Significant down-regulation of IFN-α mRNA was observed in all birds vaccinated by different methods and the vaccinated-challenged groups at 3 dpc. The significant up-regulation was observed in the Gel-ch and the ON-ch groups at 1 dpc compared to the control group. There was a low significant difference in the SP-ch compared to spray-vaccinated group at 3 dpc (Figure 5.9 B1). Comparison between the different methods of immunisation showed that the mRNA expression of IFN-α at 1 dpc was significantly higher in the Gel-ch and the ON-ch birds (Figure 5.9 B2).

IFN-β mRNA expression in the turbinate was significantly down-regulated at 3 dpc in the gel- and ON-vaccinated groups. The significantly up-regulated was seen in the spray-vaccinated birds at 5 dpc. In the vaccinated-challenged groups, there was significant down-regulation at all-time points in the Gel-ch and SP-ch groups, and at 1 and 5 dpc in the ON-ch group compared to the control group. Significant high differences were observed in the spray-vaccinated at 3 and 5 dpc compared to SP-ch groups, and at 3 dpc in the ON-ch compared to ON-vaccinated group (Figure 5.10 B1). Comparison between the different vaccination routes showed significantly higher level in expression of IFN-β at 3-5 dpc in the spray-vaccinated group, and at 1 dpc in the ON-ch group (Figure 5.10 B2).

Trachea

Expression of IFN-α was significantly down-regulated at 3 and 5 dpc in the spray-vaccinated groups and only at 3 dpc in the ON-vaccinated groups. Significant up-regulation was present at 5 dpc in both gel- and ON-vaccinated birds. In the vaccinated-challenged groups, the IFN-α was significantly down-regulated at all-time points in the Gel-ch and the SP-ch groups, the expression was significantly up-regulated only at 1 dpc in the ON-ch group compared with the control group. Significant high differences were noticed at 3 and 5 dpc in the gel-vaccinated compared to Gel-ch groups, and at 3 dpc in the ON-ch compared to ON-vaccinated groups (Figure 5.9 D1). The mRNA expression of IFN-α was significantly higher at 3-5 dpc in the gel-vaccinated group, and at all sampling days in the ON-ch (Figure 5.9 D2).

The expression of IFN-β mRNA was significantly down-regulated in all vaccinated groups at 3 dpc, followed by significant up-regulation at 5 dpc only in the ON-vaccinated birds. In the vaccinated-challenged groups, there was significant down-regulation of this gene in the Gel-ch and SP-ch at1 and 3 dpc, while in the ON-ch, IFN-β was up-regulated significantly at 5 dpc compared to the control group. Significant high differences were observed at 3 dpc in the ON-ch compared to ON-vaccinated birds (Figure 5.10 D1). The expression of IFN- β mRNA showed significantly higher at 5 dpc in the ON-vaccinated birds, and at 3 and 5 in the ON-ch group (Figure 5.10 D2).

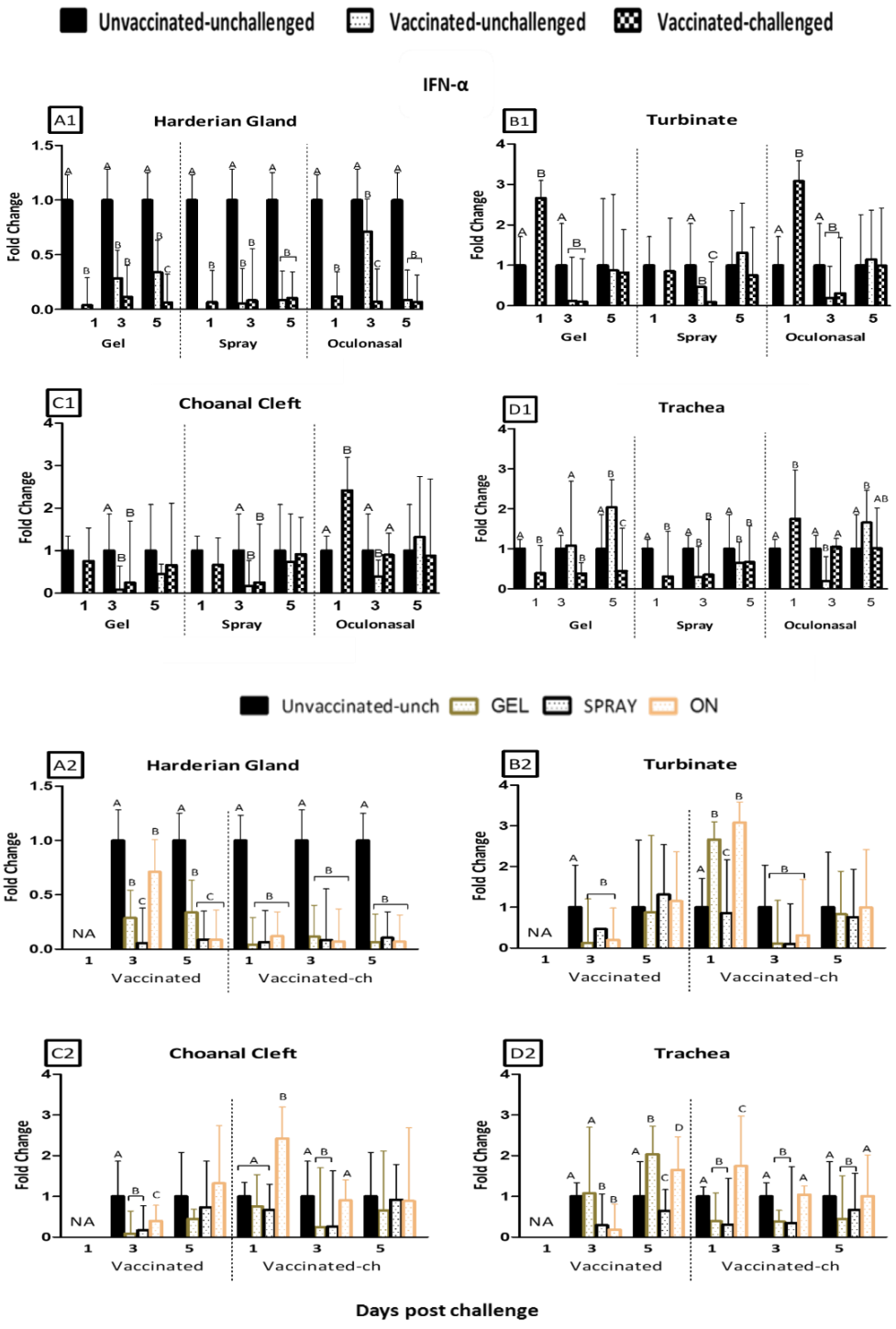

Figure 5.9. Relative mRNA expression of (A1-D1) IFN-α in the Harderian gland, turbinate, choanal cleft and trachea sample of chickens. (A2-D2) IFN-α presents a comparison between the different routes on the same tissue and day. Significant differences between the groups indicated with different letters. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected control group (n=3). Data from the vaccinated-unchallenged groups have not been processed at 1 dpc and indicated with (NA) "not available".

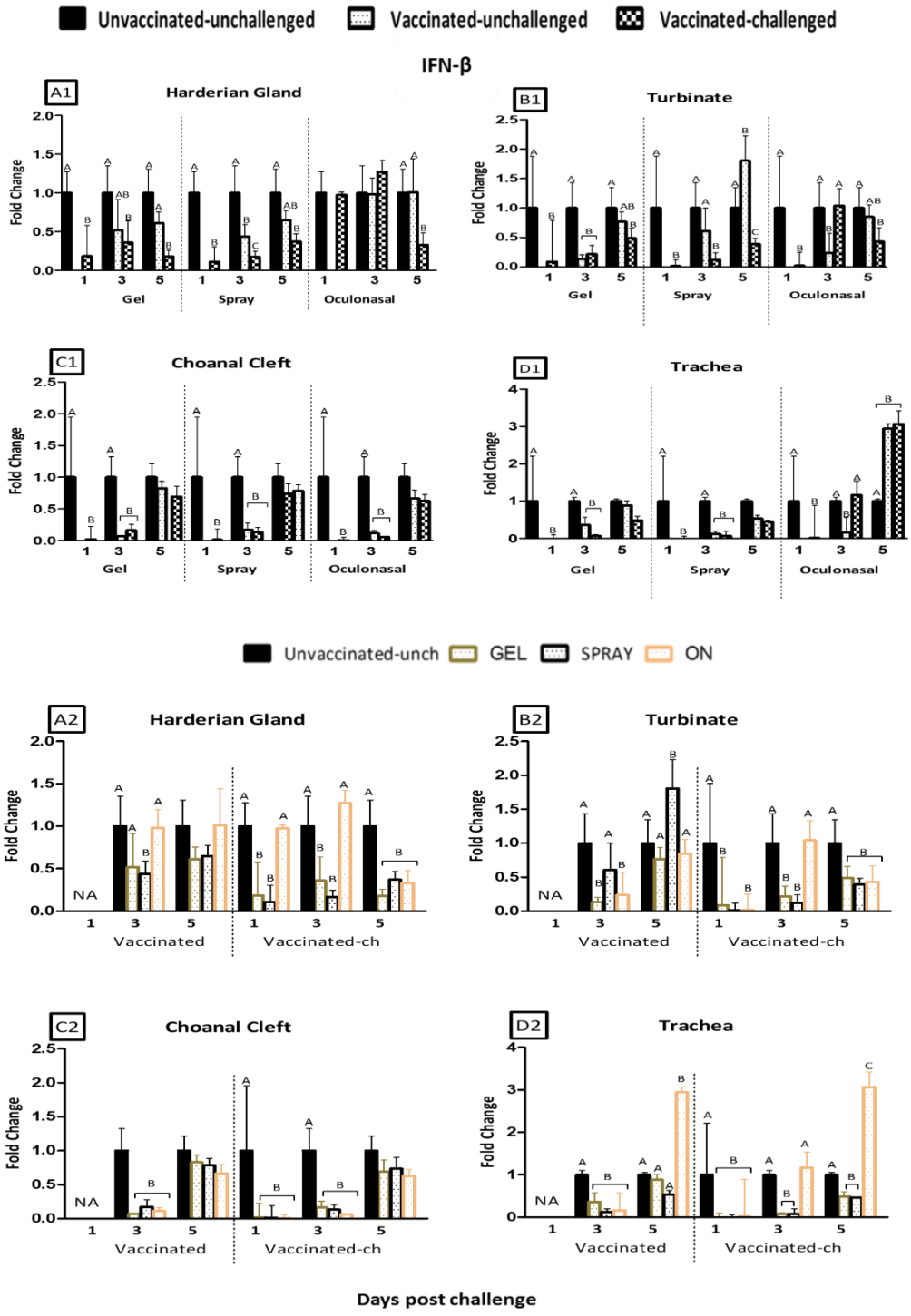

Figure 5.10. Relative mRNA expression of (A1-D1) IFN-β in the Harderian gland, turbinate, choanal cleft and trachea sample of chickens. (A2-D2) IFN-β present a comparison between the different routes on the same tissue and day. Significant differences between the groups indicated with different letters. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected control group (n=3). Data from the vaccinated-unchallenged groups have not been processed at 1 dpc and indicated with (NA) "not available".

5.3.9 Pro-inflammatory cytokine regulation (IL-6 mRNA expression)

Harderian gland (HG)

There was a significant up-regulation of the IL-6 mRNA expression at 3 and 5 dpc in the spray, and at 3 dpc in the ON-vaccinated groups. In the vaccinated-challenged groups, the IL-6 observed significantly down-regulated at 1 dpc in both Gel-ch and ON-ch groups. Whereas the SP-ch group was significantly up-regulated at 3 dpc compared to the control group. Significant high differences were noticed at 3 and 5 dpc in spray-vaccinated compared to SP-ch groups, and at 3 dpc in ON-vaccinated compared to ON-ch chickens (Figure 5.11 A1). The mRNA expression of IL-6 presented significantly higher in the spray and the ON-vaccinated birds at 3 dpc, and only at 5 dpc in the ON-vaccinated group. In addition, a significant low expression was noted at 1 dpc in the Gel-ch (Figure 5.11 A2).

Choanal cleft

There was only significant up-regulation in the mRNA expression of IL-6 in the ON-vaccinated group at 3-5 compared to the control and ON-ch groups. A significant up-regulation was present at 1 dpc in all vaccinated-challenged groups compared to the control group. (Figure 5.11 C1). The observed mRNA expression of IL-6 was significantly higher in the ON-vaccinated birds at 3 and 5 dpc compared to the other vaccination methods (Figure 5.11 C2).

Turbinate

There was significant up-regulation in the mRNA expression of IL-6 at 1 dpc in the all vaccinated-challenged groups in birds vaccinated by all methods compared to the control group, a significant down-regulation was seen at 5 dpc in ON-ch group (Figure 5.11 B1). IL-6 mRNA expression presented significantly lower in the gel-vaccinated birds

at 5 dpc, while in the vaccinated-challenged groups, IL-6 was higher at 1 dpc in the Gel-ch (Figure 5.11 B2).

Trachea

There was a significant up-regulation of the IL-6 mRNA expression at 3 dpc, followed by down-regulation at 5 dpc in the ON-vaccinated group. The significant up-regulation was noted at 1 and 3 dpc in the ON-ch, and only at 1 dpc in both Gel-ch and SP-ch groups compared to the control group. Significant high differences were noted in the ON-ch compared to ON-vaccinated groups between at 3 and 5 dpc (Figure 5.11 D1). In comparison between the different methods of vaccination, the mRNA expression of IL-6 showed significantly higher in the ON-vaccinated birds at 3 dpc, and in the Gel-ch and SP-ch groups at 1 dpc. In addition, significantly higher differences were noticed in the ON-ch at 3 and 5 dpc compared to other vaccination routes (Figure 5.11 D2).

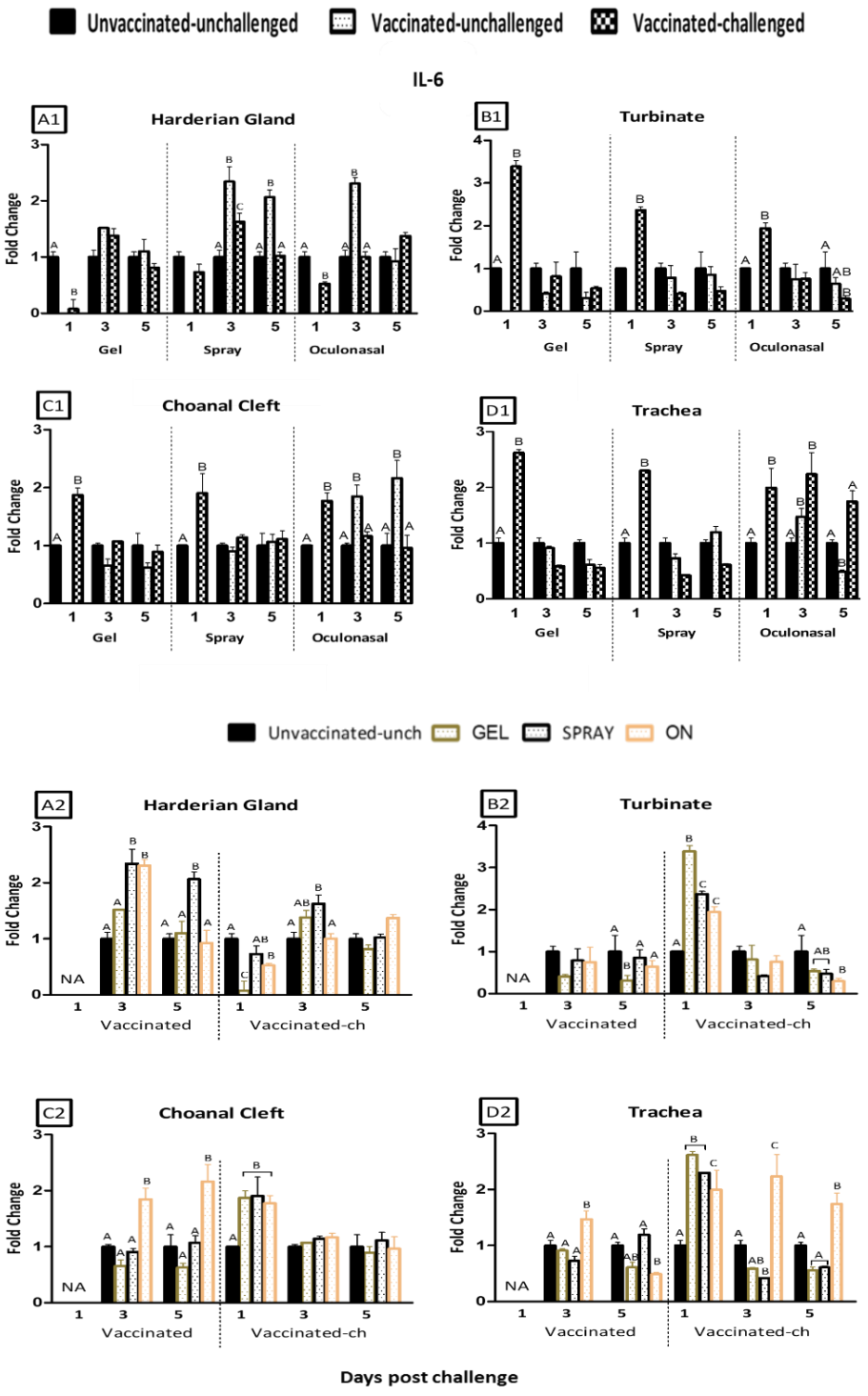

Figure 5.11. Relative mRNA expression of (A-D) IL-6 in the Harderian gland, turbinate, choanal cleft and trachea sample of chickens. (A1-D1) present a comparison between the different routes on the same tissue and day. Significant differences between the groups indicated with different letters. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected control group (n=3). Data from the vaccinated-unchallenged groups have not been processed at 1 dpc and indicated with (NA) "not available".

5.4 Discussion

Route of administration can affect IB vaccine virus localisation that may influence the early mucosal and cell-mediated immune responses and host's gene signatures. The choice of one route over the other depends on the type of vaccine, vaccine uptake, efficacy and stability of the vaccine strain under different conditions. Literature review showed that few IBV vaccine studies have addressed the effects of different routes of administration on the protection levels and host innate immune responses of broiler chickens, before and after challenge with virulent M41 (al-Tarcha *et al.* 1991; Andrade *et al.* 1983; Gough & Alexander 1979; Toro *et al.* 1997; Winterfield *et al.* 1976). This is the first study demonstrating the combined administration of Mass+793B vaccines via three different methods in commercial broiler chicks. In this Chapter, to compare the efficacy of the vaccination methods, cilia protection was evaluated, early mucosal and cell-mediated immune responses were quantified, and gene signatures in the head-associated lymphoid and respiratory tissues were examined.

Following vaccination, chicks irrespective of vaccination methods showed mild clinical respiratory signs such as head shaking, tracheal râles and snicks, which started to appear within a week following vaccination. Such reactions demonstrate that the vaccination route did not influence the onset of post-vaccinal reactions. Similar findings have been reported in day-old broiler chicks vaccinated by eye-drop, coarse spray, or intratracheal instillation with combined infectious bronchitis live vaccines containing different strain of American IBVs (Andrade *et al.* 1983).

This study demonstrated that when the vaccines of Mass+793B is simultaneously administered by Gel, Spray or ON, based on cilia-stopping test, the protection conferred against M41 challenge was above 80%, although slight advantage was found

with the ON method (94.5%). This is the first study to demonstrate that Gel-vaccination with Mass+793B provides similar protection as the spray and ON vaccinations. In exploring the reasons behind the protection, it was clear that irrespective of vaccination methods, the mucosal immunity induction as measured by anti-IBV IgA in lachrymal fluid, was almost the same in all vaccinated groups. Gough and Alexander (1979) compared the response to a Mass type vaccine of 3-week-old SPF chicks vaccinated by eye drop, spray and via drinking water, and reported similar levels of protection in all groups.

All vaccination methods showed similar patterns of systemic antibody titres that gradually decreased until they were significantly lower at 21 dpv compared to 3 dpv. The combined IB vaccines administered via different vaccination methods had an effect on maternally derived antibodies (MDA) decline, where MDA titres were reducing faster than the control but at the similar rate between the vaccinated groups. Similar findings have been reported before (Darbyshire & Peters 1985). Low levels of anti-IBV antibodies can result from the partial neutralisation of the vaccine virus by MDA present in young broilers, with an added consequence of low virus replication and poor stimulation of the humoral response (Chhabra *et al.* 2015a; Mondal & Naqi 2001). It has been previously reported that broilers can be ELISA positive, due to MDA, until levels diminish around 21 days of age (Awad 2014; de Wit & Cook 2014). The antibody titres were significantly high in the gel-vaccinated group at 10 dpv compared to other routes. This could be due to low levels neutralisation of MDA when the vaccines were given by gel compared to the ON or spray methods. It has been previously reported that the differences in the humoral antibody response following immunisation are

related to administration route and vaccination dose (Okino *et al.* 2013; Toro *et al.* 1997).

Lachrymal anti-IBV IgA and IgY plays an important role against IBV infection of the upper respiratory tract (Okino *et al.* 2013); Chapter 4). In Chapter 4, working on broilers, it was proposed anti-IBV IgA and anti-IBV IgY (in lachrymal fluid) followed by tissue IFN-α plays an essential role in conferring protection against virulent IBV challenge, and this can be included as IBV vaccine efficacy quantitative biomarker (Al-Rasheed et al., submitted). In this experiment, analysis of immune mediators, again demonstrated that the anti-IBV IgA and IgY levels at 5 dpc in the lachrymal fluid was significantly raised following the M41 challenge. There were no significant differences between the vaccinated-challenged groups reflecting almost the same levels of protective mucosal immunity in these groups. Despite variations in the viral load of HALT and respiratory tissues, the mucosal induction of immunity was similar, reflecting that minimal viral titres such as the one provided by the spray vaccination was sufficient to induce adequate IgA and IgY antibodies in lachrymal fluids. This could have been the reason for similar tracheal ciliary protection in all the vaccinated groups.

In understanding the vaccine virus intake, the viral load in the tissues were assayed, and this demonstrated significant low viral load in the trachea of the spray-vaccinated group compared to the gel and ON methods at 10-21 dpv and 3-10 dpv, respectively. This may be due to loss of virus particles because aerosolisation (lost in the environment), resulting in inadequate dose of vaccine received by each chick when applied using coarse spray (al-Tarcha *et al.* 1991). For all three routes of vaccine administration, the viral load was significantly higher in the turbinate and choanal cleft

than the trachea in all days in spray, at 3-10 dpv in gel and only at 21 dpv in ON-vaccinated groups. In a previous study, a greater viral replication was demonstrated in the nasal turbinate and trachea tissues in birds inoculated with IBV strains (Darbyshire *et al.* 1978). In addition, using *in-situ* hybridization, Dolz *et al.* (2012) demonstrated the presence of viral RNA in the nasal turbinate with extensive replication at 1 dpi, which occurred prior to infection in the trachea.

At 1, 3 and 5 dpc, the viral load in the HG and turbinate tissues in all vaccinated-unchallenged groups were either similar or greater compared to the vaccinated-challenged. However, the viral load in the trachea was significantly higher in the ON-ch group, with notably significant high differences compared to the Gel-ch and SP-ch groups. It is likely that the lymphoid tissue, such as the HG, including the lymphoid components in the turbinate (nasal-associated lymphoid tissues), were able to eliminate the virus administered via ON (Darbyshire *et al.* 1976; Darbyshire *et al.* 1978) compared to the tracheal epithelium. The qRT-PCR results of this study show that the HG in the ON- and spray-vaccination routes induced a good resistance to challenge compared to the gel-vaccinated birds. This finding is consistent with that of Ratanasethakul and Cumming (1983) who found that vaccination by the conjunctival, intranasal and in-contact routes induced a good resistance to challenge. The lower viral load in vaccinated-challenged groups indicates the influence of the combined vaccine on viral attachment and the proliferation of the challenged virus (M41).

In this study, following challenge, broadly significant up-regulation of TLR3 was noticed only in the ON-vaccinated group in the HG, Turbinate and choanal cleft. In addition, MDA5 expression showed a significant increase at 1-3 dpc for all tissues in all groups,

compared to the control. Notably, significant up-regulation of TLR3 and MDA5 was noted for the turbinate, choanal cleft and trachea in the ON-ch and Gel-ch groups compared to the SP-ch. The increase in TLR3 and MDA5 gene expression coincided with higher ciliostasis protection scores, suggesting that the ON and gel-vaccinated routes may provide a greater degree of protection compared to the spray vaccination.

The TLR3 pathway leads to type I interferon production, which has antiproliferative and antiviral properties (Kameka *et al.* 2014). IFN-α expression showed a significant increase at 1 dpc for the turbinate and trachea in Gel-ch and ON-ch groups. This also accords with our earlier observations (Chapter 4), which showed that IFN-α mRNA expressed significantly at 1 dpc in vaccinated-challenged birds compared to vaccinated-unchallenged, suggesting a useful parameter for measuring early host response to IBV in chickens. IFN-β is the end product of the TLR3 and MDA5 pathways and has been reported to protect against infection with different IBV strains, tissue tropism, histopathology and antiviral state (Chhabra *et al.* 2018; Okino *et al.* 2017). Up-regulation of IFN-β significantly peaked at 5 dpc in the turbinate and trachea in SP-unch birds and in both ON-vaccinated groups. Due to the up-regulation of IFN-β, it is likely to be considered as a central mediator, with a role in the innate immune response against IBV infection (Kint *et al.* 2015; Yang *et al.* 2018).

The current study found that the IL-6 mRNA was significantly up-regulated in all tested tissues (except HG) from vaccinated-challenged groups at 1 dpc. IL-6 inhibits the cellular immune responses generated by the vaccine, preventing the production of proinflammatory cytokines and other pathological changes. IL-6 expression in the HG from gel-vaccinated groups presented significantly lower compared to chickens

vaccinated via spray or the ON. A possible explanation for this might be that vaccines applied by spray and ON routes increase the exposure (and subsequent stimulation) of the HG. As part of the inflammation reaction, IL-6 increases are connected to viral load, tissue tropism and injuries, and particularly has been demonstrated in the trachea (Asif *et al.* 2007; Chhabra *et al.* 2018; Okino *et al.* 2017).

In conclusion, results of this study confirmed that the gel and spray methods of vaccinations induced similar levels of humoral and mucosal immunity as the ON application. In addition, findings in this study showed significant up-regulation of TLR3, MDA5, and IFN-α in the ON-vaccination group in the tissues of vaccinated-challenged compared to the vaccinated-unchallenged chickens. Although, the ON route remains as the optimal method for primary vaccination of broiler chickens, this study has demonstrated that both spray and gel vaccinations could equally provide sufficient levels of protection against IBV M41. Further investigations are needed to compare these methods of immunisation of chicks under the field condition.

Chapter 6: Early host immune response in layer hens following vaccination by drinking water or oculonasal routes

Abstract

Host innate immune responses in head-associated lymphoid tissues (HALT) and respiratory tissues were evaluated in laying hens following monovalent vaccination with either Mass (H120) or 793B (4/91) serotypes. Each vaccine was applied to two groups, either by drinking water (DW) or oculonasal (ON) routes. At 1, 3, 5 and 14 days post vaccination (dpv), Harderian gland (HG), turbinate, choanal cleft, and trachea tissues were processed for viral load and host mRNA expressions. Blood and lachrymal fluid were taken at 7 and 14 dpv to determine anti-IBV antibody titres by ELISA. With exception of DW-H120 group, it was found that lachrymal IgY was significantly greater at 14 dpv compared to control groups. Viral RNA load in the HG and turbinate was significantly higher in the ON-H120 group compared to all other vaccinated groups at 1–3 dpv. Host TLR3, MDA5, and IL-6 mRNA were significantly up-regulated in the HG at 3–5 dpv in the ON-H120 and ON-4/91-vaccinated groups, respectively compared to control and all other groups. Expression of IFN-β in the HG and trachea was up-regulated in both DW and ON H120-vaccinated groups compared to control group. Expression of CD8α and CD8β indicated high up-regulation at 1–3 dpv in the turbinate and at 3–5 in the trachea compared to the control group. Furthermore, IgA mRNA was expressed significantly in the ON-H120 and ON-4/91- vaccinated groups compared to DW-vaccinated groups. Generally, mucosal and cellular immune response was greater in the turbinate than the trachea for both methods of vaccination. The current study is the first empirical investigation into the impact of IBV vaccine interactions in adult layers by monitoring innate immune responses (TLR3, MDA5, CD8α, CD8β, IgA, and IgY). Data demonstrated the variable role of innate, mucosal and cellular immunities in relation to vaccine type and route of administration.

6.1 Introduction

Clinical signs in layers resulting from infectious bronchitis virus (IBV) may be associated with high mortality and nephritis and reduced egg production and lower quality eggshells and albumen in layer hens, leading to economic losses for poultry farmers (DEFRA 2005; Hewson *et al.* 2014). The prevention of IB in chicken layers is achieved through the use of inactivated and live vaccines, which provide protection against virulent field IB viruses in the event of an exposure. Substantial economic problems in poultry flocks can be problematic to control by vaccination, depending on the endemic strains (Sjaak de Wit *et al.* 2011), and outbreaks of IB frequently occur in commercial layers (Gelb *et al.* 1991; Liu & Kong 2004).

Live attenuated viral vaccines are commonly used to induce local immunity, indicating that local mucosal immunity is crucial for induction of effective defence against this disease (Chhabra *et al.* 2015b; Guo *et al.* 2008; Okino *et al.* 2013). Vaccines are generally administered to layer pullets to achieve early protection, and adult layer hens and breeders are boosted with inactivated vaccines at point of lay at 13 to 18 weeks of age (Bande *et al.* 2015; Box & Ellis 1985; Finney *et al.* 1990), which vary based on their similarity to the circulating field viruses (Sjaak de Wit *et al.* 2011). Establishing an effective vaccination schedule against IB has been difficult due to the high level of antigenic variation among circulating viruses (Cook *et al.* 2012). This difficulty emphasises the importance of understanding different aspects of immunity following live vaccine administration in layers to reduce economic losses.

The Massachusetts serotype IBV vaccine is used throughout the world, as it was the first IBV vaccine produced and the only one available for many years (Jackwood 2012; Jordan 2017). The Massachusetts H120 vaccine virus has been used successfully for the

initial vaccination of layers and breeders (Bijlenga *et al.* 2004). Another serotype which is well distributed in Europe, Asia, and other parts of the word is 793B (Worthington *et al.* 2008). Strains belonging to the 793B serotype include 4/91 or CR88, that emerged in the 1990s associated with major welfare and economic problems in apparently well-vaccinated flocks (Gough *et al.* 1992; Parsons *et al.* 1992).

After IB vaccination, systemic, mucosal, humoral, and cell-mediated immunity (CMI) are stimulated (Chhabra *et al.* 2015b; Okino *et al.* 2013; Smialek *et al.* 2017; Smialek *et al.* 2016). However, all previous studies were carried out in SPF or broiler chickens. With regards to early host immune responses to IBV in egg- laying chickens, there has been no prior publications available.

Many researchers outlined different categories of innate immune response often observed in either IBV vaccination or infection, such as TLRs, MDA5, type I interferons, and pro-inflammatory cytokines (Chhabra *et al.* 2018; Okino *et al.* 2013; Okino *et al.* 2017; Smialek *et al.* 2016). To date, no studies have addressed the immune response in layer hens by measuring host gene signatures, mucosal, cellular and humoral immunity in the HALT and respiratory tissues. This study investigates the mucosal, innate, cellular and humoral immune responses following administration of Mass (H120) or 793B (4/91) live vaccines either by drinking water or oculonasal routes.

6.2 Materials and methods

6.2.1 Layer chickens

Ninety 41-week-old light brown Lohman layer chickens were obtained from a commercial farm and kept up to three weeks according to animal welfare guidelines under strict biosecurity measures in the University of Liverpool (Chapter 2.3). The hens

were reared on deep litter with nest boxes, antibiotic-free water and feed, which were provided *ad libitum* (Chapter 2.4).

6.2.2 Attenuated live IBV vaccines

Two commercial live IBV vaccines were administered in this study, Massachusetts serotype (H120, Boehringer Ingelheim Animal Health Limited, UK) and 793B serotype (4/91, MSD Animal Health, South Africa). Vaccines were stored at 4 °C until required and then dissolved in sterile distilled water (SDW) according to the manufacturer's instructions. Each hen received 100 µl of vaccine or SDW via the oculonasal (ON) route or via oral inoculation (DW) according to the experimental design. Each vaccine strain was titrated in TOCs using the Reed-Muench method (Reed & Muench 1938) (Chapter 2.2). The titre of the vaccine viruses used was 3.75 $\log_{10}$ $TCID_{50}$/ml and 4.45 $\log_{10}$ $TCID_{50}$/ml for the H120 strain and 4/91 strain, respectively.

6.2.3 Experimental design

Ninety 41-week-old layer chickens were divided into six groups (n = 15 per group) and distributed as follows (Table 6.1); (A1) drinking water (DW)-unvaccinated control, (A2) oculonasal (ON)-unvaccinated control, (B1) DW-H120, (B2) ON-H120, (C1) DW-4/91 and (C2) ON-4/91. Groups A1 and A2 were sham-inoculated with 0.1 ml of chilled vaccine-free SDW via DW and ON methods, respectively. Layer hens in groups (B1 and C1) were vaccinated via DW and chickens in groups (B2 and C2) vaccinated by ON routes with the volume as recommended by the manufacturer (0.1 ml) of live H120 at dosage of ($10^{3.75}$ $CD/_{50}$) and 4/91 at dosage of ($10^{4.45}$ $CD/_{50}$) vaccines at 42 weeks of age. Before vaccination, all hens were monitored daily for one week for egg production and clinical signs before vaccination, and oropharyngeal swabs (OP) swabs were collected at one week before hens arrive to confirm an absence of IBV. Following vaccination,

OP and cloacal swabs (CL) were collected from five chickens at 1, 3, 5, 7, and 14 dpv for virus detection and quantification by qRT-PCR. At 7 and 14 dpi, lachrymal fluid was collected to assay for anti-IBV antibodies by monoclonal ELISA, and blood was collected via the brachial vein from five birds in each group, serum was separated and stored at -20 °C. Three birds from each group were humanely euthanized at 1, 3, 5, and 14 dpv. The Harderian gland, turbinate, choanal cleft, and trachea were collected and stored at -20 °C in RNA*Later* (Qiagen, Crawley, UK) for determination of viral load and host gene expression analysis by quantitative real-time RT-PCR (qRT-PCR).

Table 6.1 Study design showing groups and vaccine programs. At 42 weeks of age, 15 hens from each group were vaccinated with H120 or 4/91 or sham-inoculated with SDW.

Group	Vaccination methods (n)
A. Unvaccinated	(A1) Sham vaccinated by DW (15) (A2) Sham vaccinated by ON (15)
B. H120-vaccinated	(B1) vaccinated by DW (15) (B2) vaccinated by ON (15)
C. 4/91-vaccinated	(C1) vaccinated by DW (15) (C2) vaccinated by ON (15)

6.2.4 Systemic humoral antibody titres

Blood samples from the brachial vein were collected from five birds of each group at 7 and 14 dpv and placed in labelled 5 ml bijou tubes without coagulant overnight at room temperature. Serum was stored at -20 °C until further use (Chapter 2.8.2).

6.2.5 Lachrymal fluid IgA and IgY titres

Lachrymal fluids were collected at 7 and 14 dpv using sodium chloride and immediately centrifuged at 3,000 x g for 5 mins before the supernatant was stored at -20 °C as previously described (Ganapathy *et al.* 2005) (Chapter 2.8.3).

6.2.6 Serology

6.2.6.1 IBV ELISA titres

A commercial IBV ELISA kit (IDEXX, Westbrook, Maine, USA) was used to determine anti-IBV antibodies for all groups according to the manufacturer's guidelines, with a positive ELISA titre cut-off determined to be 396 (Chapter 2.18.1).

6.2.6.2 Measurement of IBV-specific IgA and IgY in lachrymal fluid

Lachrymal fluid for IBV-specific IgA and IgY at 7 and 14 dpv was assayed using an indirect ELISA (Dhinakar Raj & Jones 1996c; Ganapathy *et al.* 2005; Mockett & Cook 1986), Chapter 2.18.2.

6.2.7 Swab samples

Five OP and CL swabs were taken from each group on all sampling days. Swabs from the same group were pooled, dipped into 1.5 ml of guanidine thiocyanate (solution D), and stored at - 70 °C until required (Chapter 2.8.1).

6.2.8 Molecular detection of IBV

6.2.8.1 Extraction of RNA (swab)

RNA was extracted from pooled OP and CL swabs using the QIAamp viral RNA mini kit (Qiagen, UK) following the manufacturer's instructions (Chapter 2.12.2).

6.2.8.2 Extraction of RNA (tissues)

Total RNA was extracted from collected tissues using the RNeasy Plus Mini Kit (Qiagen, UK) according to the manufacturer's instructions (Chapter 2.13).

6.2.9 Real-time RT-PCR

Viral load was quantified from extracted RNA in HG, choanal cleft, turbinate, and trachea tissues, and swabs by quantitative real-time RT-PCR (qRT-PCR) using IBV 3'untranslated region (UTR) gene-specific primers and probes (Ball *et al.* 2016b; Chhabra *et al.* 2018; Jones *et al.* 2011) (Chapter 2.20).

6.2.10 Host gene expression analysis

Host gene expression analysis was carried out for samples collected at 1, 3, and 5 dpv. QRT-PCR of cDNA samples was performed in triplicate using LightCycler 480 SYBR Green I Master mix and primers (Chapter 2.21). Data were normalised against 18S ribosomal RNA expression using a relative standard curve method (Chhabra *et al.* 2018; Kuchipudi *et al.* 2012; Okino *et al.* 2013; Rajesh *et al.* 2016). For CD8α, CD8β, IgA, and IgY, the calculation of fold change was carried out as previously investigated using 18S rRNA as a reference gene (Livak & Schmittgen 2001; Okino *et al.* 2013; Okino *et al.* 2017), and data presented as fold change difference in gene expression of vaccinated against unvaccinated control group.

Table 6.2. Primers used in the qRT-PCR analysis for relative quantification of gene expression.

Gene Group	Gene Target	Primer sequences: Forward (F) and reverse (R)	Reference
1. Reference gene	18S ribosomal RNA	(F) TGTGCCGCTAGAGGTGAAATT (R) TGGCAAATGCTTTCGCTTT	(Kuchipudi et al. 2012; Okino et al. 2013)
2. Cellular immune response	CD8α	(F) TTG GAC GGG ACC TTA CAG AC (R) TGA AGG GAG CAA AGG AGA AA	(Okino et al. 2013)
	CD8β	(F) CTGCATGGCTCCGACAATGG (R) ATCGACCACGTCAAGCTGGG	(Okino et al. 2017)
3. Mucosal immune response	ChIgA (Chicken immunoglobulin A)	(F) TGCAGGGCAATGAGTTCGTCTGTA (R) AGGAGGTCACTTTGGAGGTGAAT	(Ndegwa et al. 2012)
	ChIgY (Chicken immunoglobulin Y)	(F) GACGAAGCTT TTCCTCTTCT (R) CCCGATTGTA CCCTCTATCG	(Zheng et al. 2001)
4. Viral recognition	TLR3 (Toll-like receptor 3)	(F) GCAATTTCTCCTTCACCTTTTCA (R) CCTTTATGTTTGCTATGTT-GTTATTGCT	(Kuchipudi et al. 2014)
	MDA5 (Melanoma differentiation-associated protein 5)	(F) AGGAGGACGACCACGATCTCT (R) CCACCTGTCTGGTCTGCATGT	(Kuchipudi et al. 2014)
5. Interferon	IFN-β (Interferon beta (Type I))	(F) TCCAACACCTCTTCAACATGCT (R) TGGCGTGTGCGGTCAAT	(Kuchipudi et al. 2014)
6. Inflammation	IL-6 (Interleukin 6)	(F) CACGATCCGGCAGATGGT (R) TGGGCGGCCGAGTCT	(Kuchipudi et al. 2014)

6.2.11 Statistical analysis

Data were analysed using either student's t-test (two groups) or one-way analysis of variance (ANOVA), followed by a post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00. Differences between groups were considered significant at ($P<0.05$) (Chapter 2.22).

6.3 Results

6.3.1 Humoral anti-IBV specific antibody ELISA titres

High levels of IBV antibody were detected in the control groups at 7 and 14 dpv, with the mean antibody titres being 4970±759 and 4062±218, respectively. At 7 dpv, a significantly higher titre was observed in the DW-4/91 group (8065±792) in comparison with the control and DW-H120 groups. At 14 dpv, there was a significantly higher

antibody titre in the DW-4/91 group (8076±556) compared with the control and H120-vaccinated groups via same route. Significantly higher differences in the antibody titre were observed at 14 dpv in hens vaccinated with H120 (8306±636) or 4/91 (7999±461) via the ON method compared to the DW-H120 group and control group (Figure 6.1).

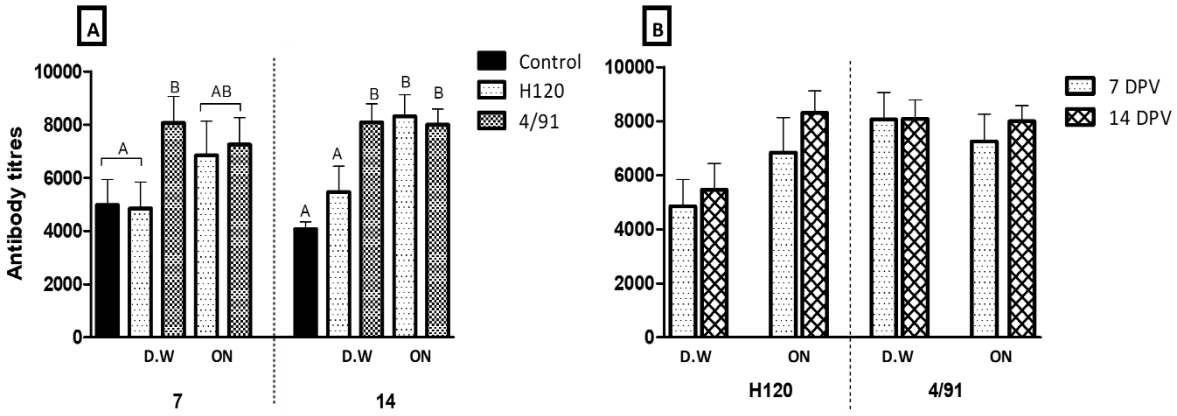

Figure 6.1. Mean anti-IBV ELISA antibody titres of the control and vaccinated groups at 7 and 14 dpv. Data are expressed as mean values ± SEM (n=5). The ELISA cut-off is 396. Significant differences between the groups were detected by one-way ANOVA (A) and t-test (B). Different letters indicate significant differences within groups.

6.3.2 Local immunity

6.3.2.1 Determination of IBV-specific lachrymal IgA titres using monoclonal ELISA

The level of specific IgA anti-IBV in lachrymal fluids in unvaccinated birds was 0.283 at 7 dpv. The level of specific anti-IBV IgA was low in all vaccinated birds at 7 dpv (DW and ON-H120 groups (0.334 and 0.495), DW and ON-4/91 hens (0.264 and 0.239). The IgA antibody titres peaked at 14 dpv in all H120 immunised (DW = 0.744 and ON = 0.572), and 4/91 immunised hens (DW = 0.436 and ON = 0.514). A significantly high difference in IgA levels was only observed in the DW-H120 group at 14 dpv compared to the

control (Figure 6.2 A), or when compared to 7 dpv (Figure 6.2 B). No significant differences were found between chickens vaccinated with both strains via the different routes (Figure 6.2 A).

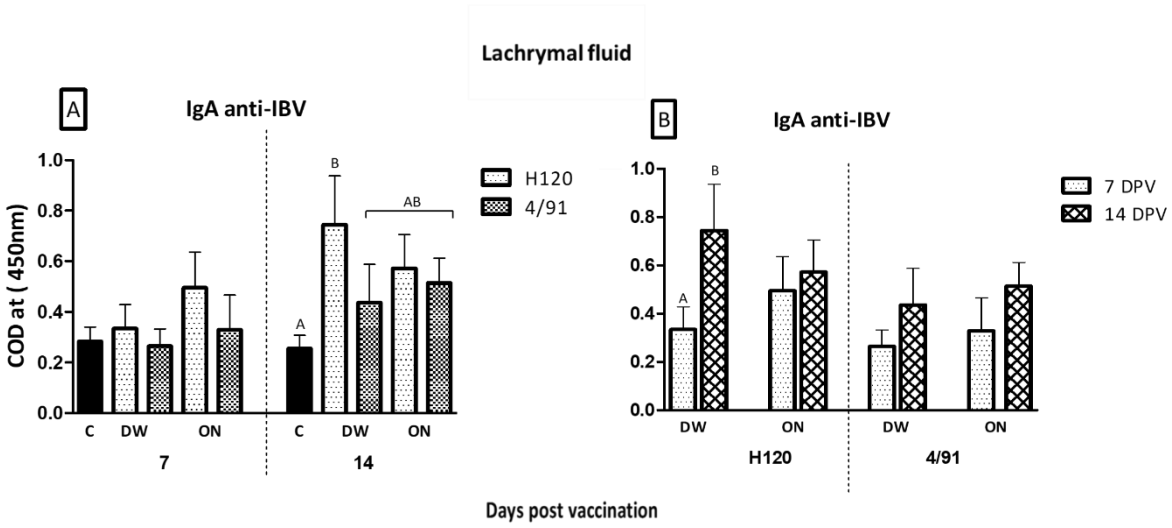

Figure 6.2. Measurement of IBV-specific IgA titre using monoclonal indirect ELISA from the lachrymal fluid. Mean IgA from lachrymal fluids collected at 7 and 14 dpv (n=5). Data are expressed as mean of corrected optical density (COD). Different letters indicate significant differences within groups. Significant differences were determined using one-way ANOVA (A) and t-test (B).

6.3.2.2 mRNA expression of IgA in the turbinate and trachea

Turbinate

In both ON-vaccinated groups, there was significant up-regulation in the mRNA expression of IgA in the turbinate at all-time points compared with DW-vaccinated chickens, with the exception of H120-vaccinated group at 1 dpv. In the ON-4/91 group, there was significant up-regulation of IgA mRNA expression at 1 dpv compared to the ON-H120 vaccinated group (Figure 6.3 A).

Trachea

There was significant up-regulation of IgA mRNA expression in the trachea of both ON-vaccinated groups at 3 and 5 dpv compared to DW-vaccinated hens. In the ON-4/91 group, there was significant up-regulation of IgA mRNA expression at 1 and 3 dpv compared to ON-H120 vaccinated group. At 5 dpv, the mRNA expression of IgA was significantly up-regulated in the ON-H120 group compared to the ON-4/91 vaccinated birds (Figure 6.3 B).

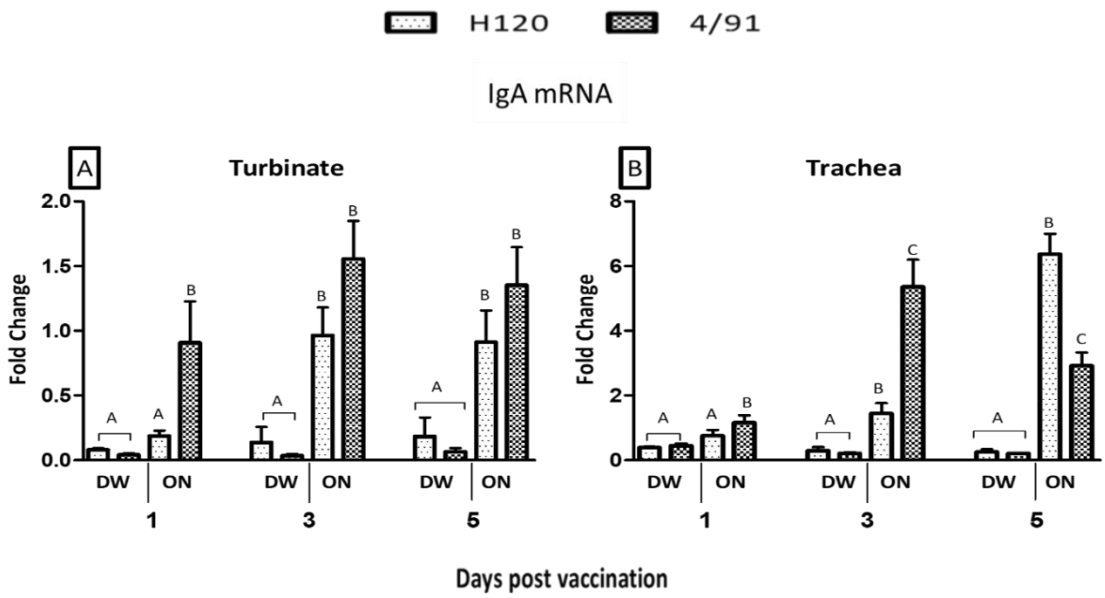

Figure 6.3. Relative mRNA expression of IgA in the turbinate and tracheal tissues at 1, 3, and 5 dpv. Data are expressed as fold change compared to the control group (n=3). The relative expression of IgA is calculated based on 'double delta Ct' (ΔΔCt) values. Different letters indicate significant differences within groups. Significant differences were determined using one-way ANOVA.

6.3.2.3 Measurement of specific lachrymal antibody (IgY) anti-IBV using monoclonal ELISA

The level of specific IgY anti-IBV in lachrymal fluids in unvaccinated birds was 1.647 at 7 dpv. The level of specific anti-IBV IgY in vaccinated chickens via both methods was detected at 7 and peaked at 14 dpv [DW and ON-H120 groups (1.843 and 2.030), and DW and ON-4/91 hens (2.150 and 2.023)]. In 4/91-vaccinated hens (DW and ON) and in ON-H120 vaccinated birds, IgY titres were observed to be significantly higher at 14 dpv compared to the control group. The DW-4/91 group was significantly higher in comparison to the DW-H120 group at 7dpv (Figure 6.4 A).

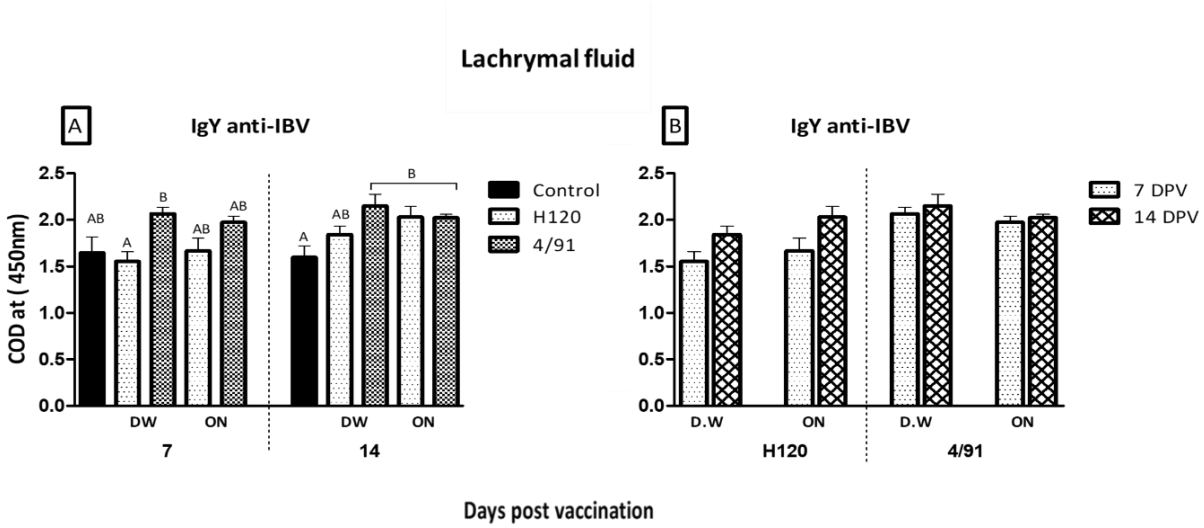

Figure 6.4. Measurement of IBV-specific IgY titre using monoclonal indirect ELISA from the lachrymal fluid. Mean IgY from lachrymal fluids collected at 7 and 14 dpv (n=5), data expressed as mean of corrected optical density (COD). Different letters indicate significant differences within groups. Significant differences were determined using one-way ANOVA (A) and T-test (B).

6.3.2.4 mRNA expression of IgY in the turbinate and trachea

Turbinate

There was significant up-regulation of IgY mRNA expression in the turbinate in all vaccinated groups compared to control. There was significant up-regulation of IgY mRNA expression at 3 dpv in the ON-4/91-vaccinated group in comparison to both H120-vaccinated groups. Moreover, expression of IgY in birds vaccinated via DW showed higher significant expression at 5 dpv in 4/91-vaccinated birds compared with the H120-vaccinated group (Figure 6.5 A).

Trachea

In all vaccinated groups, up-regulation of IgY mRNA expression was observed in trachea through different methods compared to control group. No statistical differences were noted in both strain groups immunised by both methods (Figure 6.5 B).

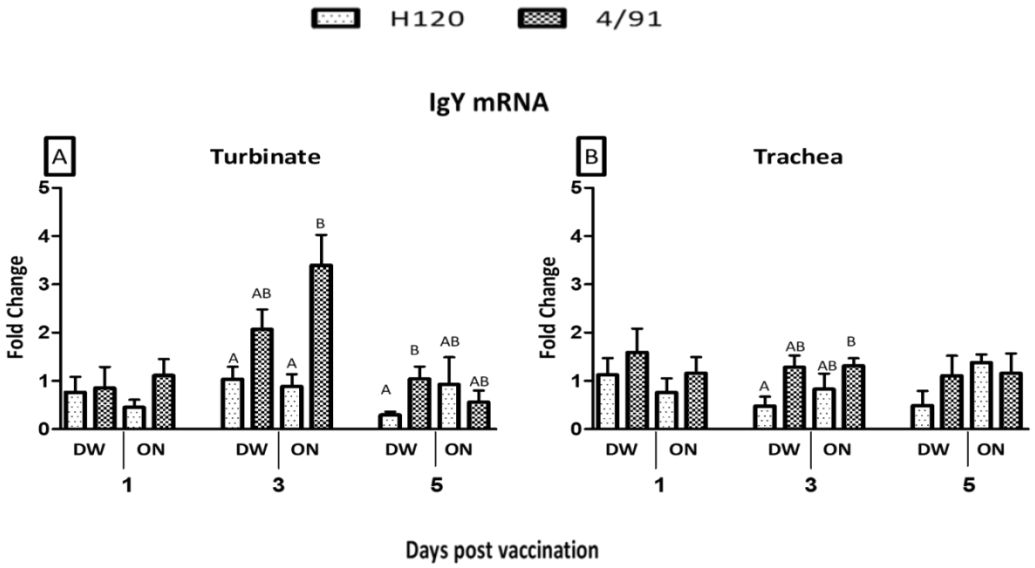

Figure 6.5. Relative mRNA expression of IgY in the turbinate and tracheal tissues at 1, 3, and 5 dpv. The relative expression of IgY is calculated based on 'double delta Ct' (ΔΔCt) values. Data are expressed as fold change compared to the control group (n=3). Different letters indicate significant differences within groups. Significant differences were determined using one-way ANOVA.

6.3.3 Viral RNA quantification

6.3.3.1 Pooled swabs

Viral RNA loads in all swabs in the control group were below the detection limit on all dpv (Figure 6.6). The viral RNA load in OP swabs peaked at 3 dpv in the DW-vaccinated birds for H120 and 4/91 (2.49 log REU and 3.46 log REU, respectively), while the viral RNA load in both ON vaccinated groups peaked at 7 dpv (3.99 log REU and 3.91 log REU, respectively) (Figure 6.6 A). For the CL swabs, the viral RNA load peaked earlier at 1 dpv in the DW-vaccinated birds for H120 and 4/91 (3.74 log REU and 2.81 log REU, respectively), whereas the viral RNA load in both ON vaccinated birds peaked later at 14 and 7 dpv (3.94 log REU and 2.64 log REU, respectively) (Figure 6.6 B).

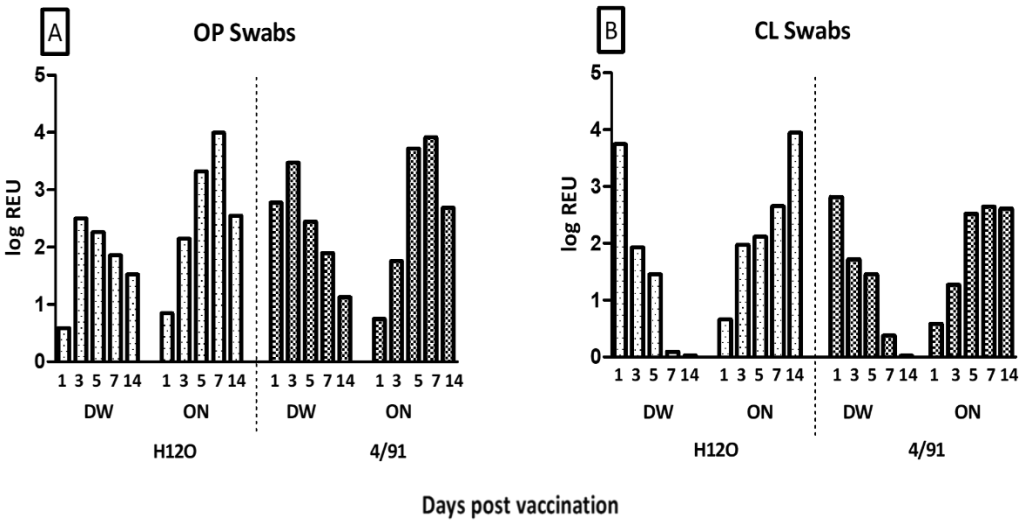

Figure 6.6. Quantification of viral RNA expressed as log-relative equivalent units (REUs) of RNA. (A) and (B) IBV loads at 1, 3, 5, 7, and 14 dpv in the pooled OP and CL swabs, respectively. Data presented as mean (n=5); chickens were vaccinated with IBV strains of H120 or 4/91 at a dose of $10^{3.75}$ CD_{50} /bird and $10^{4.45}$ CD_{50}/bird, respectively.

6.3.3.2 Tissues

Harderian Gland (HG)

Viral load in the HG peaked significantly at 3 dpv compared to other sampling points in both groups (H120 and 4/91) vaccinated via ON. In chickens vaccinated by DW, the viral copy peaked at 5 dpv, and a significantly higher level was seen in H120-vaccinated birds compared to other sampling days. Both 4/91 vaccinated groups and the ON-H120 group were negative at 14 dpv (Figure 6.7 A1). There was significantly higher viral RNA load in the ON-H120-vaccinated birds in the HG at 1 and 3 dpv in comparison to the other groups. A statistically higher difference was seen in DW-vaccinated chickens with both strains at 5 dpv compared to the ON-vaccinated chickens (Figure 6.7 A2).

Choanal cleft

Viral RNA level was detected on all sampling days, except at 14 dpv, in both vaccinated groups. The viral titre was significantly higher at 3 dpv than all the other sampling days in the H120-vaccinated group via both routes. However, in the 4/91-vaccinated groups, the viral copy peaked significantly only in ON-4/91 group at 3 dpv compared to the other time points (Figure 6.7 C1). Significantly higher virus levels were noted at 5 dpv in DW-H120 and DW-4/91 vaccinated birds compared to the ON-4/91-vaccinated birds (Figure 6.7 C2).

Turbinate

Viral RNA was detected on all sampling days, except 14 dpv, in all vaccinated groups. Viral RNA load in both strain groups peaked significantly in birds vaccinated by both methods at 3 dpv in comparison to the other time points (Figure 6.7 B1). There were significantly higher viral loads in the ON-H120-vaccinated birds at 1 dpv compared to all other groups, and only at 3 dpv compared to DW-4/91 group. Moreover, the viral

titre in the turbinate was statistically higher in both strain groups in DW compared to the ON-vaccinated group at 5 dpv (Figure 6.7 B2).

Trachea

Viral RNA load was detected on all sampling days, except at 14 dpv, in the ON-vaccinated group. The viral copy peaked significantly at 3 dpv in ON-vaccinated chickens for both strains and in DW-4/91 group compared to other sampling days (Figure 6.7 D1). Furthermore, the ON-H120-vaccinated birds was significantly lower at 1 and 5 dpv compared to other groups. (Figure 6.7 D2).

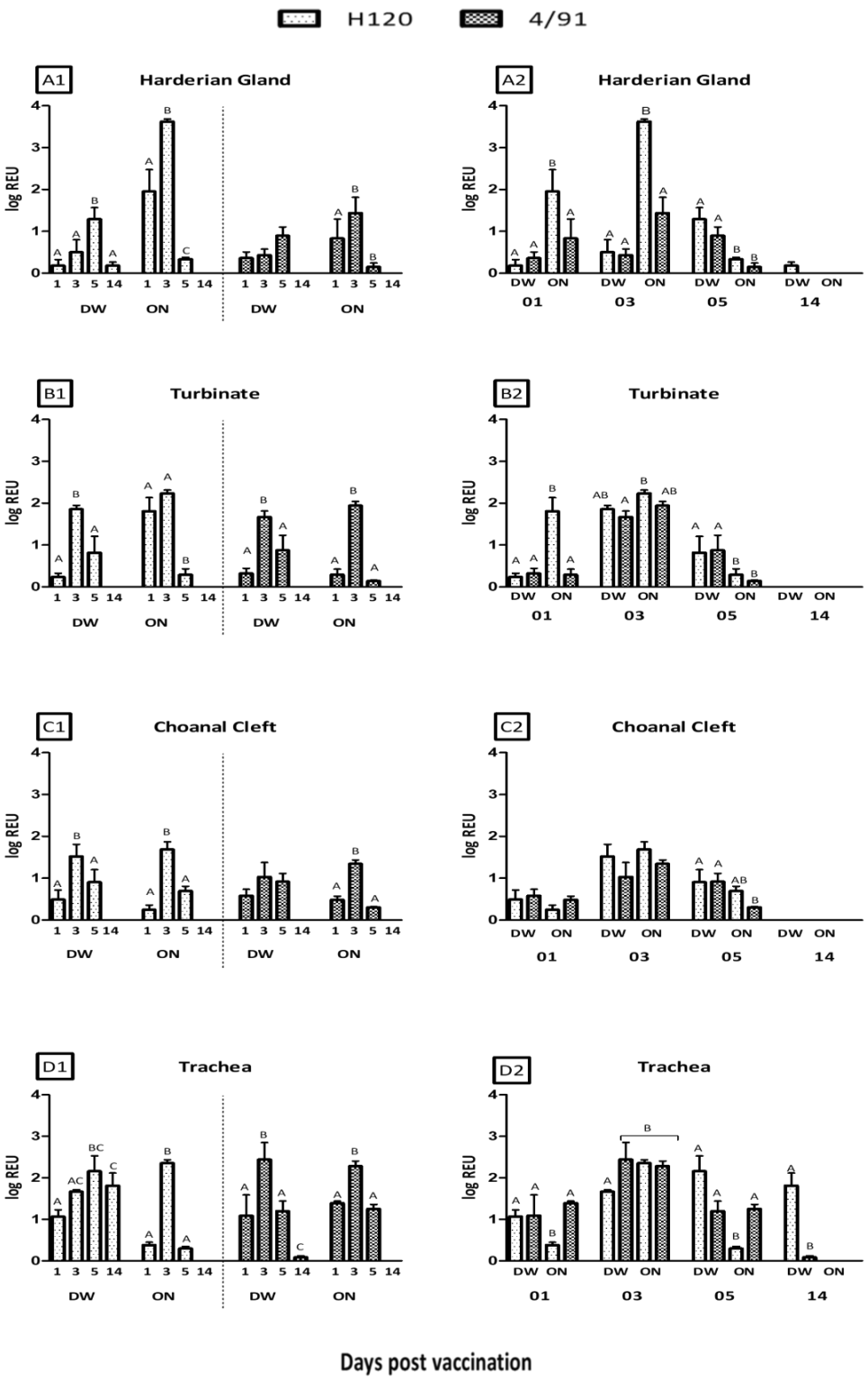

Figure 6.7. Quantification of viral RNA expressed as a log REU of RNA in the HG, turbinate, choanal cleft, and trachea samples of chicken (dpv). Layers were vaccinated with IBV strains of H120 or 4/91 at a dose of $10^{3.75}$ CD_{50} /bird and $10^{4.45}$ CD_{50}/bird, respectively. Different letters indicate significant ($P<0.05$) differences within groups (n=3). Significant differences were determined using one-way ANOVA.

6.3.4 Expression of TLR3 and MDA5 mRNA

Harderian gland (HG)

There was significant up-regulation in the mRNA expression of TLR3 in H120-vaccinated birds via both routes of vaccination at 3 dpv, and at 5 dpv in DW-4/91 and ON-H120-vaccinated birds compared to other groups (Figure 6.8 A). Expression of MDA5 showed significant up-regulation at 3 and 5 dpv in the ON-H120-vaccinated birds via both the DW- and ON-vaccination routes compared to other groups (Figure 6.8 E).

Choanal cleft

A significant down-regulation was observed in the mRNA expression of TLR3 in the 4/91-vaccinated group at 3 dpv for birds vaccinated by DW and at 5 dpv in both vaccinated methods compared to the control group (Figure 6.8 C). For expression of MDA5, results indicated significant down-regulation at 5 dpv in the DW-4/91-vaccinated group compared to the ON-H120-vaccinated group and the control group (Figure 6.8 G).

Turbinate

Significant down-regulation was noted in the mRNA expression of TLR3 at 1 and 3 dpv in DW-vaccinated chickens of the H120- and 4/91-strain groups, respectively, compared to other groups. This was followed by significant down-regulation in all groups at 5 dpv compared to the control group (Figure 6.8 B). There was significant down-regulation of MDA5 gene expression at 1 dpv in the DW-4/91 and ON-H120-vaccinated birds compared with the control group (Figure 6.8 F).

Trachea

There was significant up-regulation in the mRNA expression of TLR3 at 5 dpv in DW-4/91 and ON-H120-vaccinated birds compared with the control group (Figure 6.8 D). Expression of MDA5 showed significant up-regulation at 5 dpv in H120-vaccinated chickens via both immunisation routes compared with the control group (Figure 6.8 H).

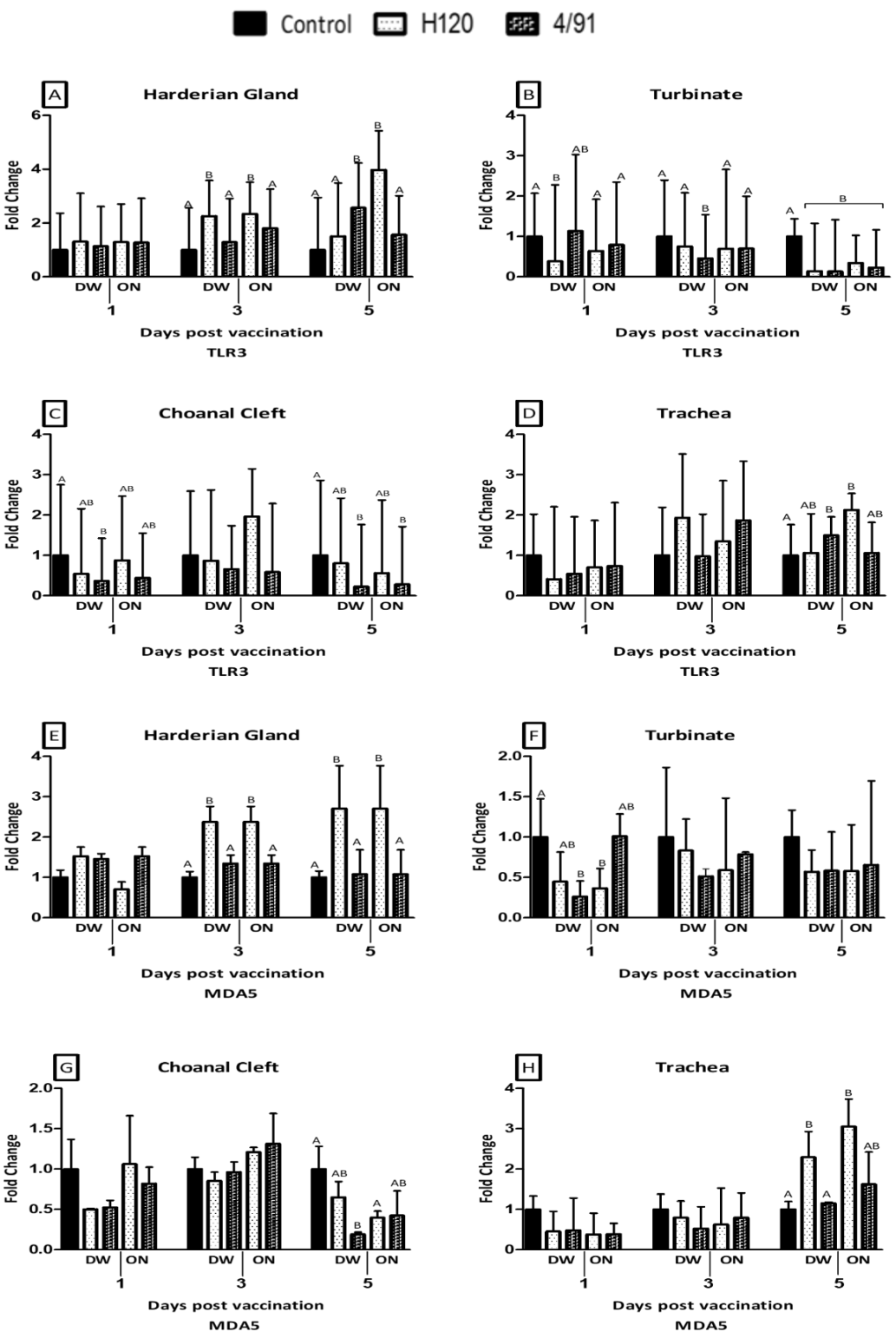

Figure 6.8. Relative mRNA expression of TLR3 (A-D) and MDA5 (E-H) in the Harderian gland, turbinate, choanal cleft, and trachea sample of layer chickens. Significant differences between the groups are indicated with different letters ($P<0.05$). Data represent the mean with error bars as standard error and are expressed as fold change relative to the unvaccinated control group (n=3).

6.3.5 Expression of host IFN-β mRNA

Harderian gland (HG)

There was significant up-regulation of IFN-β at all-time points in ON-H120-vaccinated birds compared to the other groups, and at 1 and 3 dpv in DW-H120-vaccinated birds compared with the control group. In the 4/91-vaccinated group, significant up-regulation of IFN-β mRNA expression was observed in DW at 1 dpv and at 3 dpv in the ON-vaccinated groups compared with the control group, with statistically high changes noted compared to other groups (Figure 6.9 A).

Choanal cleft

There was only significant up-regulation in the mRNA expression of IFN-β at 3 dpv in the ON-H120 group compared to other groups. No other significant differences were observed in any vaccinated groups (Figure 6.9 C).

Turbinate

There was significant down-regulation of IFN-β mRNA expression in the H120-vaccinated group in both vaccination routes and in the DW-4/91 group at 1 dpv, followed by significant down-regulation in all groups at 5 dpv compared to the control group. Significant up-regulation of IFN-β mRNA was observed in the DW-H120 group at 3 dpv. No statistical differences were observed between groups (Figure 6.9 B).

Trachea

Expression of IFN-β mRNA was significantly up-regulated in DW-vaccinated chickens with both strain groups at 3 and 5 dpv and only at 5 dpv in ON-vaccinated birds with both strain groups compared to the control group (Figure 6.9 D).

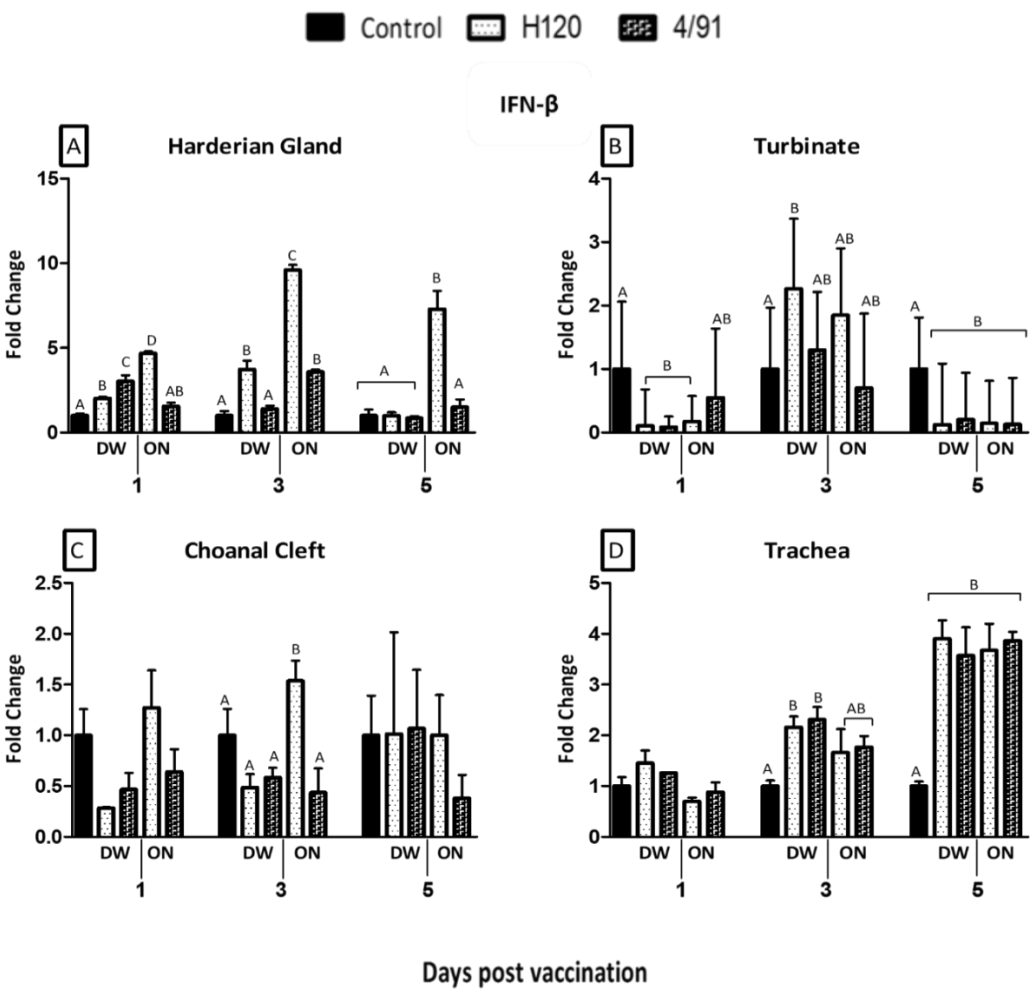

Figure 6.9. Relative mRNA expression of IFN-β (A-D) in the Harderian gland, turbinate, choanal cleft, and trachea sample of chickens. Significant (P<0.05) differences between the groups are indicated with different letters. Data represent the mean with error bars as standard error and are expressed as fold change relative to the unvaccinated control group (n=3). Significant differences between the groups were detected by one-way ANOVA.

6.3.6 Pro-inflammatory cytokine (IL-6) regulation

Harderian gland (HG)

There was significant up-regulation of IL-6 mRNA expression at 3 dpv in the ON-4/91 group compared to other groups, with the exception of DW-H120-vaccinated birds. The DW-4/91 group, IL-6 was significantly up-regulated at 5 dpv compared to other groups (Figure 6.10 A).

Choanal cleft

No significant changes were noted in any groups (Figure 6.10 C).

Turbinate

There was significant up-regulation of IL-6 at 3 dpv in the DW-H120-vaccinated group compared to other groups, with the exception of the ON-4/91-vaccinated birds. At 5 dpv, there was significant down-regulation of IL-6 mRNA expression in the DW-4/91- and ON-H120-vaccinated groups compared to other groups (Figure 6.10 B).

Trachea

Expression of IL-6 mRNA was significantly up-regulated in ON-H120-vaccinated chickens at 3 and 5 dpv compared with the control group. In the 4/91-vaccinated group, there were only significant up-regulation at 3 dpv in the DW-vaccinated birds compared with the control group (Figure 6.10 D).

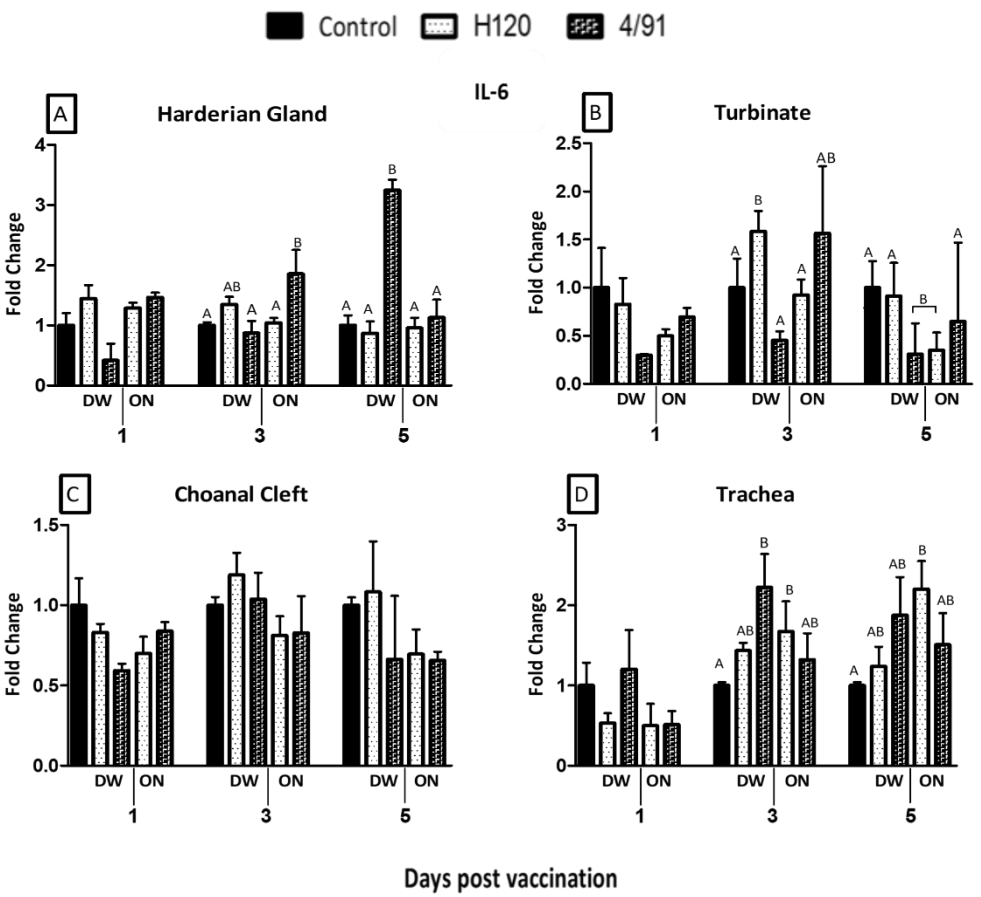

Figure 6.10. Transcription profile of proinflammatory cytokine IL-6 (A-D) in the Harderian gland, turbinate, choanal cleft, and trachea sample of chickens. Significant ($P<0.05$) differences between the groups are indicated with different letters. Data represent the mean with error bars as standard error and are expressed as fold change relative to the unvaccinated control group (n=3). Significant differences between the groups were detected by one-way ANOVA.

6.3.7 Cell-mediated immune responses (mRNA expression of CD8α and CD8β)

Turbinate

Expression of CD8α and CD8β mRNA was significantly up-regulation in all vaccinated groups on all sampling days compared with the control group. There was significant up-regulation of CD8α mRNA expression at 3 dpv in DW-H120 group compared to other groups (Figure 6.11 A). Compared to other groups, birds in the ON-4/91 group demonstrated significantly higher CD8β mRNA expression at 1 dpv (Figure 6.11 C).

Trachea

A significant up-regulation of CD8α and CD8β mRNA was noted in all vaccinated groups on all sampling days compared with the control group. Significant up-regulation of CD8α was noted at 3 dpv in ON-H120 group compared to 4/91-immunised birds via both vaccination methods (Figure 6.11 B). Expression of CD8β mRNA was significantly up-regulated only at 5 dpv in the ON-H120 group compared to other groups (Figure 6.11 D).

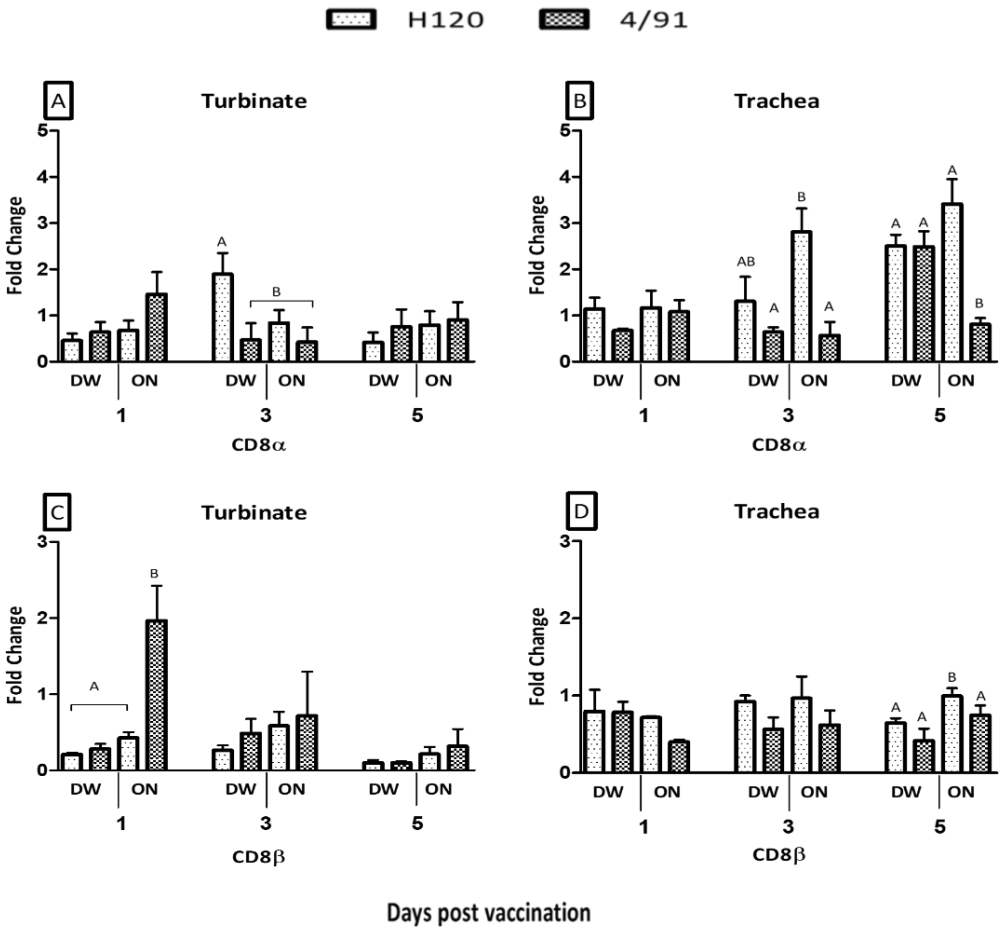

Figure 6.11. Cell-mediated immune responses. (A-B) Relative mRNA expression of CD8α and (C-D) relative mRNA expression of CD8β genes in the turbinate and trachea tissues samples. The relative expression of CD8 is calculated based on 'double delta Ct' (ΔΔCt) values. Data represent the mean with error bars as standard error and are expressed as fold change relative to the unvaccinated control group (n=3). Significant differences between the groups indicated with different letters ($P<0.05$).

6.4 Discussion

Live attenuated IBV vaccines (such as 4/91 and H120) are used extensively in chicken flocks to control IBV infection, disease and production losses. There has not yet been detailed investigation of the immune response in the head-associated lymphoid (HALT) and respiratory tissues following IBV vaccination in layer hens. This is the first study conducted to investigate the hen's innate, mucosal, cellular and immune responses following immunisation with a live IBV vaccine of either H120 or 4/91. In addition, the innate response to different vaccination methods was also compared.

Layers in the unvaccinated control group had a high titre of anti-IBV ELISA antibodies at 42 weeks of age. All birds in the study received IBV live vaccines at the rearing stage and inactivated IB vaccines at 15 - 18 weeks of age (Bande *et al.* 2015; Box & Ellis 1985; Finney *et al.* 1990). In this study, it appears that the re-administration of either live Mass or 793B vaccines induced increased levels of humoral antibody titres in comparison to the controls. Although live vaccines of IB have been shown to neutralise humoral antibodies (de Wit 2000; Mondal & Naqi 2001), no such impact was evident in the adult egg-laying hens used in this study. However, at 14 dpv, for vaccinated groups other than DW-H120, there was a substantial increase in humoral antibody titres. Although IB humoral antibodies levels have little to no correlation with respiratory protection (Cavanagh 2007; Cook *et al.* 2012), it has been shown that blood antibody levels have a significant influence in terms of providing protection against a drop in egg production and quality (Box *et al.* 1988).

IgA antibodies plays an important role in mucosal immunity to IBV. Its presence in lachrymal secretions following IBV antigen inoculation has been previously reported (Cook *et al.* 1992; Toro & Fernandez 1994). In this study, mucosal immunity was

assessed by detecting the IgA and IgY of the lachrymal fluid and the early changes in the turbinate and trachea by assaying the mRNA expression of IgA and IgY. For lachrymal IgA, no significant increase was found, with the exception of the DW-H120 group (at 14 dpv). In young chickens, previous studies have reported increased levels of IgA and IgY in lachrymal fluid following ocular and/or DW vaccination with H120 (Okino *et al.* 2013; Toro *et al.* 1997) and Ma5 and/or 4/91 via the ON route (Smialek *et al.* 2017), and high levels of IBV-specific IgA have been associated with a degree of protection against virulent IBVs (Okino *et al.* 2013).

In Chapter 3, it was demonstrated that an equivalent viral load and replication of IBV occurs in the turbinate and trachea. Therefore, it was hypothesised that similar levels of mucosal immunity could exist in these tissues. When the vaccines were given through the ON route, a significantly higher level of mRNA in IgA was found in the turbinate and trachea of both vaccinated groups in comparison to the controls. In this study, between the vaccinated groups, there were no significant differences in the mRNA of IgA the turbinate. In the trachea, a significantly higher level of IgA mRNA expression was found in the 4/91 group. This demonstrates that early induction of mucosal immunity in the turbinate and trachea tissues can be assessed by measuring IgA mRNA, particularly when the vaccines were administered via the oculonasal route. Oculonasal inoculation seemingly results in a robust local immunity presence in the HG originating from higher IBV exposure compared to the oral inoculation. This suggests the importance of the HG in contributing to local immunity through IgA production. These results elucidated the unique immune properties of the HG, a local immune tissue that must quickly respond to vaccines that enter via the eye (Deist & Lamont 2018). Parallel to previous findings in broiler birds (Chapter 5), the ON-vaccination

method is the most effective route for producing a local immune response in the HG in layer chickens. These results are similar to those found by Gallego *et al.* (1992), who demonstrated that soluble antigen administration in seven-week-old chickens via the ocular route is the most efficient route for producing a local immune response in the HG.

In Chapters 3 and 4, it was demonstrated that following on from the M41-infection of unvaccinated and vaccinated broiler chicks, one of the major findings was the induction of IBV-specific lachrymal IgA and IgY, and IgA levels is associated with protection (Chhabra *et al.* 2015b; Da Silva *et al.* 2017; Okino *et al.* 2013). Monoclonal ELISA data demonstrated that by 14 dpv, a significantly higher level of lachrymal IgY was found in DW-4/91, ON-H120 and ON-4/91. This corresponds with the increased humoral antibody titres of these groups. Guo *et al.* (2008); Okino *et al.* (2013); Orr-Burks *et al.* (2014) emphasised that IgY is more efficient at neutralising antigens than IgA, thus the increased levels of IBV-specific IgY would be beneficial in minimising infection, disease and production losses. Analysis of trachea and turbinate IgY mRNA production provides evidence of almost two times significant up-regulation in the turbinate, particularly for the ON-4/91 group. It appears that the early analysis of the mRNA of IgY following virulent or attenuated IBV infection in these respiratory tissues can provide further evidence of vaccine uptake.

Viral load patterns of live vaccines following administration in egg-laying hens has not been reported before. In both the OP and Cl swabs, viral load increased or declined depending on the route of vaccination (DW or ON), irrespective of the vaccine strains. As reported before (de Wit & Cook 2014; Toro *et al.* 1997), it appears that ON

vaccination presents the viruses to the replication sites better when compared to the DW route. For the tissues, viral load patterns differed depending on the strain and route of administration. Within the ON-H120 vaccinated group, it appears that the viral load in the HG, turbinate, choanal cleft and trachea increased and peaked at 3 dpv. When the same vaccine was given by the DW route, replication appeared to be delayed depending on the tissue. It was also noted that viral load was significantly higher at 1-3 dpv in the HG and turbinate and at 3 dpv in the trachea of birds that received the vaccine by ON rather than the DW route. This finding is consistent with those of Darbyshire *et al.* (1976) who demonstrated that positive antigen levels in the turbinate have been observed between 1–3 dpc after vaccination with IBV H120. In addition, using *in-situ* hybridization, Dolz *et al.* (2012) demonstrated the presence of viral RNA in the nasal turbinate with extensive replication at 1 dpi, before trachea becomes positive.

In the trachea at 3 and 5-14 dpv, the viral load of DW-4/91 and H120 were both significantly higher against the other groups. It appears that in the trachea, H120 replicates slowly but persistently. When the vaccines were given oculonasally, H120 replication in the HG and turbinate and frequently had significantly higher titres when compared to 4/91. Some of these strains, including the live Mass type IB H120 strain, appear to have the upper respiratory tract as the principle target (Toro *et al.* 1997). In the trachea, similar or higher viral loads of 4/91 were detected compared to H120. This reflects the specific characteristics of the vaccines such as the predilection to certain tissues and the evasion of the immune response (Cavanagh & Naqi 2003).

Gene signatures of TLR3 and MDA5 were up-regulated in the HG at 3 and 5 dpv following ON inoculation. For all of these time points, significantly higher fold changes were found in the ON-H120-vaccinated hens in contrast to the ON-4/91 group. Significantly higher levels of fold change were found in the HG-TLR3 and MDA5 (3 and 5 dpv), and trachea-MDA5 (5 dpv) in the H120-vaccinated groups. Thus, it is demonstrated that the stimulation of a gene signature is dependent on the route of vaccination, and likely to be influenced by the tissue tropism and replication potential of the virus strains. There was delayed up-regulation of TLR3 in the HG and the turbinate of 4/91 vaccinated birds reflecting a delayed attachment, replication and recognition by the host. Interestingly, TLR3 and MDA5 expression in DW-H120 hens were both significantly higher when compared to the DW-4/91 group. This possibly explains the better induction of lachrymal IgA in this group (see above).

Compared to the control, all tissues other than in the turbinate, showed a significantly higher expression of IFN-β when either vaccine was administered via ON or DW. The response was robust in the ON groups rather in than the DW groups, again reflecting the higher availability of the vaccine viruses in the tissues compared to the DW administration. At 3 dpv, in the turbinate, there was a significant up-regulation of IFN-β only in the DW-H120 group. The reason for this is unclear. In Chapter 4, findings in broiler chickens showed that IFN-β expression down-regulated at 1-2 dpc followed by up-regulation at 4-5 dpc. The TLR3 pathways are stimulated by all IBVs, however such stimulation is more pronounced for virulent strains that initiate a greater production of IFN-β through TIR-domain-containing adapter-inducing interferon-β (TRIF) (He *et al.* 2016).

The pro-inflammatory cytokine (IL-6) showed higher up-regulation in the HG at 3 dpv in the ON-4/91 group and at 5 dpv in the DW-4/91 group. In addition, the expression of IL-6 was significantly up-regulated in the DW-H120 group at 3 dpv in the turbinate and at 3- 5 dpv in the trachea in the ON-H120 group compared to the other groups. This is connected to higher viral loads in the tested tissues. For IBV, high expression of IL-6 have been associated with high viral load and increased tracheal tissue damages (Asif *et al.* 2007; Chhabra *et al.* 2015b; Okino *et al.* 2014).

Although cell-mediated immunity (CMI) is an essential part of the immune response to IBV, there is little or no information available on the CMI responses following live IB vaccination in egg-laying hens. In this study, both groups receiving H120 demonstrated significantly raised CD8α and CD8β in the turbinate at 3 dpv and trachea at 3-5 dpv. It has been reported that the CD8α protein is involved in IBV clearance (Kameka *et al.* 2014; Okino *et al.* 2014; Smialek *et al.* 2017). Meanwhile, CD8β mRNA is considered to be a marker of the effector activity of CD8 T cells induced by IBV vaccination in the tracheal tissue (Okino *et al.* 2013). The mRNA from CD8+ could be used as an early marker for CD8 T-cell production due to IBV replication in the turbinate and tracheal tissues. Although T and B-cells can be assessed by IHC (Awad *et al.* 2016c; Chhabra *et al.* 2015b), the measurement of mRNA provides quantitative, fast and reliable results. In this study, by measuring the mRNA expression of CD8α and CD8β, it was evident that H120 compared to 4/91 has shown a better induction of CMI although it would have been ideal to include markers for the other T and B-cells. Due to time and cost constraints, the current investigation focused on CD8α and CD8β, and future works could consider including markers of other T-cells such as CD3, CD4 and T cell receptors (TCR) (Chhabra *et al.* 2015a; Wang *et al.* 2006).

In conclusion, this study represents the first investigation to focus on evaluating the host immune response of the head-associated lymphoid tissues (HALT) and respiratory tissues in egg-laying hens following the delivery of IBV Mass (H120) and 793B (4/91) vaccines through either the DW or ON route. A significantly higher level of IgA mRNA was observed in the turbinate and trachea of both ON-vaccinated groups, demonstrating early induction of mucosal immunity in these tissues. IBV vaccines (H120 and 4/91) actively replicate in the HALT and respiratory tissues, and based-on viral load and immune responses, the pattern of replication differs according the tissue type, vaccine-type strain, and the route of vaccine administration. An up-regulated expression of the CMI-genes was detected, which could be used as an early marker for CD8 T cell production induced by IBV immunisation. Essentially, it was demonstrated that ON vaccination, 'the gold-standard route of vaccine administration', has been shown to have advantages in terms of vaccine virus replication and the induction of immunity in laying hens. In comparison, the DW method of vaccination has shown a considerably close achievement to that of the ON. Further research should investigate the HALT and respiratory tissues in relation to the immunobiology and immunopathology of IBV in layer hens following use of other IBV strains (eg. D274, 793B (1/96 or 4/91), B48) given by the different vaccination routes, and to test the vaccinal immunity with heterologous and homologous virulent IBV challenges.

Chapter 7: General discussion and future work

Infectious bronchitis virus (IBV) is a highly contagious pathogen that causes respiratory, renal and reproductive diseases in chickens in broiler and layer chicken flocks, depending on the tropism of respective IBV strains (Cavanagh 2005b). It causes major economic losses and welfare issues in the poultry industry worldwide (Cavanagh 2007). Vaccination has been considered the most cost-effective approach to reduce or avoid losses due to IBV infection (Meeusen *et al.* 2007). However, current vaccines and vaccination programmes often fail to provide full protection, particularly with the emergence of new IBV variants (de Wit *et al.* 2011a; de Wit 2000; Jackwood 2012). In examining the protective immunities against virulent IBVs, very little information is available regarding the role of head-associated lymphoid tissues (HALT). Hence, a series of studies were undertaken in this thesis to evaluate the mucosal, humoral and cellular immunities in the HALT in chicks administered with either virulent or vaccine IBV viruses. For comparison, trachea was sampled alongside the HALT, as trachea was considered as tissue of choice for pathogenesis and vaccine efficacy evaluation.

The first two studies (Chapter 3 and 4) report for the first time, the effects of classical virulent IBV M41 on early immune responses in head-associated lymphoid and respiratory tissues in unvaccinated (Chapter 3) or vaccinated (Chapter 4) IBV MDA-positive chicks. For Chapter 3, data indicated that M41-challenge induces early upper respiratory mucosal immunity in susceptible chicks, by examining early production of lachrymal IgA following M41-infection, data demonstrated significantly higher levels at 4-5 dpc in the unvaccinated-challenged birds compared to the control. Data shows that induction of IBV-specific IgA in unvaccinated-challenged birds could be used as an indication of exposure to IBVs, although such application would require further investigation.

Though the trachea is an important tissue for IBV replication, tissues further up the respiratory tract, such as the turbinate, infraorbital sinuses and choanal cleft, in addition to HG, are at risk from exposure to air-borne infection. It was demonstrated that other than the trachea, there was high virus load in the HG, choanal cleft and turbinate. Results in this study demonstrated greatest replication of IBV at 2-3 dpc, at similar levels in all four tissues, which also was associated with the peak viral load.

The mRNA of the TLR3, MDA5, IFN-α, IFN-β and IL-6 genes were assayed. While previous work only focused on innate immune responses in the trachea (Chhabra *et al.* 2018; He *et al.* 2016; Kameka *et al.* 2014; Okino *et al.* 2017; Okino *et al.* 2014; Wang *et al.* 2006). In this study, the gene expression was carried out for lymphoid (HG, choanal cleft and pharyngeal tissue) and respiratory (turbinate and trachea) tissues. This is the first study which has attempted to examine the daily kinetics of early innate immune responses in five different tissues in M41-infected chickens. A clear pattern of mRNA up-regulation of all genes examined was found in the choanal cleft, pharyngeal, turbinate and trachea tissues, except at 1 dpc where IFN-β was down-regulated in all tissues. Tracheal expression of mRNA was similar to previous work (Chhabra *et al.* 2018; He *et al.* 2016; Okino *et al.* 2017), but in contrast to findings of Wang *et al.* (2006) who found that TLR3 expression increased at 3 dpi following M41 challenge. Due to a lack of comparative data in the literature, further research could investigate the effect of variant IBVs (such as 793B, QX and Q1) on HALT and respiratory tissues. In addition, experimental investigations may be needed to estimate the effect of co-infections with multiple pathogens (not just IBV). IBV M41 was chosen as the challenge virus to mimic one of the most common field and vaccine strain, however in order to compare between serotypes, other common IBVs such as QX and 793B should be included in

future work. Moreover, inclusion of advance molecular and immunological laboratory tools such as transcriptomics or microarray assays to identify a wider range of gene signatures would be worthwhile in further examining early immune responses in these tissues. Thus, the gene is no longer limited and after that the use of ELISA or IHC to identify where molecules are regulated within tissues.

After establishing the host immune response in naïve chickens, it is important to evaluate the same parameters in IBV-vaccinated birds vaccinated (Chapter 4). This would be useful to ascertain any potential immune parameters that could be used as an early quantitative indicator of IBV protection for vaccine efficacy studies. Current evaluation of IBV vaccine efficacy by the European Pharmacopoeia heavily relies on cilia-stopping test, which intended to demonstrate protection of cilia in vaccinated-challenged compared to cilia destruction in unvaccinated-challenged birds. However, this test relies on subjective scoring of cilia readings and provides qualitative assessment. In Chapter 4, the M41-challenge of vaccinated birds significantly increased the lachrymal IBV-specific IgA levels from 3 dpc onward, which was associated with the degree of protection against virulent IBVs (Chhabra *et al.* 2015b; Da Silva *et al.* 2017; Okino *et al.* 2013). Previous studies have not reported such change, as no sampling were carried out by or before 5 dpc, including the lachrymal fluids (Chhabra *et al.* 2015b; Da Silva *et al.* 2017; Okino *et al.* 2013). For the first time, statistically, it was proven that lachrymal anti-IBV IgA and IgY can be used as a quantitative assay for assessment of IBV vaccine efficacy.

The trachea had significant viral load in the vaccinated-challenged chickens compared to the unvaccinated-challenged at 1-4 dpc. It is likely that the lymphoid tissues,

including the lymphoid components in the turbinate (nasal-associated lymphoid tissues), were capable of eliminating the virus (Darbyshire *et al.* 1976; Darbyshire *et al.* 1978) compared to the tracheal epithelium. It appears that at least for the M41-challenge, the trachea is a better tissue of choice to demonstrate protection against challenge (Chhabra *et al.* 2015b; Okino *et al.* 2013). Unlike the cilia-stopping test, viral load provides quantitative values for a statistical comparison, and should be considered as a key indicator in future IBV vaccine efficacy studies.

Findings in this study showed significant up-regulation of TLR3 and MDA5 in the HG, turbinate and choanal cleft of the vaccinated-challenged compared to the vaccinated-unchallenged chickens. These quantifiable differences between challenged and unchallenged chickens could be used as a marker for assessing protection against IBV. Also, IFN-α expression showed a significant increase at 1 dpc for the choanal cleft, turbinate and trachea in vaccinated-challenged birds compared to vaccinated-unchallenged, suggesting a useful parameter for measuring early host response. Further studies will be needed to ascertain responses of other IBV challenge strains, as well as the inclusion of a wider range of gene signatures, inflammatory and proinflammatory cytokines and chemokines. Such comprehensive work would provide the most appropriate biomarker(s) for measurement of IBV vaccine efficacy.

In Chapter 5, a study was conducted to examine how different delivery methods of combined vaccines (H120+CR88), followed by challenge with IBV M41 impacted protection and host response. Based on cilia activity data, the protection levels of the gel-challenged (Gel-ch) and the SP-challenged (SP-ch) groups were lower than the ON-challenged (ON-ch) group. Here, it was demonstrated that all three methods of

vaccination were efficient in delivering the minimal doses needed for induction of protection against the challenge dose of M41 used in this study. Results showed that lachrymal anti-IBV IgA and IgY was detected significantly higher at 5 dpc in all vaccinated groups compared to the unvaccinated-unchallenged control group. A significantly higher IgA response following spray or ocular inoculation, given the more direct contact of the antigen with respiratory airways and HALT, such as the Harderian gland (Okino *et al.* 2013; Toro *et al.* 1997). As the Gel administration of vaccine provides similar levels of immune induction and protection, this route of vaccination could be considered for wider use in the near future. Using live IBV alongside other live viral, bacterial and coccidial vaccines could be investigated, not only in commercial chicks, perhaps also in free-range poultry.

In Chapter 6, the purpose of the study was to investigate the innate, mucosal, cellular and humoral immune responses in HALT and respiratory tissues following administration of single IBV attenuated vaccines (H120) or (4/91) given via DW or ON routes in commercial table egg layer chickens. This study provides a comprehensive evaluation of the interactions between the host (laying chickens) and the virus (IBV vaccines) through a detailed analysis of the innate immune responses, which never been attempted before in egg laying birds. Levels of humoral antibody titres in comparison to the unvaccinated controls significantly increased at 14 dpv following to the administration of either live Mass or 793B vaccines. Results showed a significantly higher level of IgA and IgY mRNA in the turbinate and trachea of both vaccinated groups via ON. This early induction of mucosal immunity in these tissues after immunisation with an attenuated vaccine through the ON route can be assessed using molecular tools. Innate immune responses represented by TLR3 and MDA5, and cytokines were

significantly up-regulated in layer chickens after IBV H120 or 4/91-vaccination. It is demonstrated that stimulation of gene signatures is dependent on the vaccine-type strain and the route of vaccine administration. In addition, Higher CD8α expression coincides with the decrease of viral load after 3 dpv in the trachea were observed. It has been reported that CD8 alpha is involved in the early stage of IBV infection during the elimination of virus-infected cells (Kameka *et al.* 2014; Okino *et al.* 2014; Smialek *et al.* 2017). Future work could consider additional genes to include the markers of other T-cells, such as CD3, CD4 and T cell receptors (TCR) (Chhabra *et al.* 2015a; Wang *et al.* 2006). With increasing practice of using live IBV vaccines in egg laying period in poultry farms, it is essential that the immunopathogenesis of these vaccines are fully understood for better induction of immunity and protection against classical and emerging variant IBVs. In addition, the characteristics of the vaccine or 'reverted' vaccines could be studied in the future.

In summary, the research chapters presented in this thesis has provided evidences that other than trachea, the head-associated lymphoid and respiratory tissues, due to its proximity to the upper air passages, play an important role in the infectivity and immune induction against IBVs. An essential finding was the role of anti-IBV IgA and IgY in the lachrymal fluid as a quantitative IBV vaccine efficacy biomarker. Also, highlighted were the role of gene signatures, type 1 interferons and cytokines following virulent or vaccine inoculation of young and adult chickens. A comparison of live attenuated IBV vaccines and routes of administration in broilers and egg-producing hens have not been examined thoroughly before. In the present project, understanding how vaccine uptake and processed, and the host innate, humoral, and cellular immune responses were outlined.

Study limitations:

- Using a single housekeeping gene (18S ribosomal RNA) for all tissues, future studies should include other reference genes such as HPRT1, ACTB, GAPDH and HMBS as studied by (Batra et al. 2017) to study their stability in different tissue types investigated for the first time such as HG, turbinate and choanal cleft.
- Another limitation is that qRT-PCR assay used in the current study for the host gene signatures, inflammatory and pro-inflammatory cytokines. With advancing in the molecular laboratories, tools such as transcriptomics or proteomic analysis for a wider range of genes could provide the most appropriate biomarker(s) for IBV vaccine efficacy models in future studies.
- This study was limited by the absence of assays to confirm the increase in mRNA expression. Assays as a Western blot or monoclonal ELISA can also analyse and confirm gene expression by directly measuring protein levels and can be used in future studies. However, this depend on the availability of standardised protocols and kits. Other than IFN gamma, commercial monoclonal ELISA kits are not available yet for all kinds of chicken's host genes signatures and cytokines.
- An additional limitation is quantification of viral RNA load without infectious viral load. Although qPCR assay detects the viral genome not infectious virus, many laboratories still choose this technique as long as the limitations are understood because qPCR is inexpensive and fast alternative with high sensitivity and specificity.

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