Studies on variant infectious bronchitis viruses: *In vitro* and *in vivo* comparison of virulence, immunopathogenesis and protection

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Abstract

Infectious bronchitis virus (IBV) variants have wide tissue tropism and high pathogenicity which provides a unique opportunity to dissect the molecular mechanisms contributing these differences. After infection of chicken embryo kidney (CEK) cells infected with IBV strains 885, QX and M41, to establish the role of host response signature to envisage the tissue tropism and pathogenicity, the apoptosis and innate immune responses were investigated. The CEK cells infected with 885 and QX shown higher cell death rate than M41. These differences in cell death rate were due to higher levels of apoptosis induction by 885 and QX as compare to M41. We found that up-regulation of toll like receptor 3 (TLR3), melanoma differentiation associated protein 5 (MDA5) and interferon beta (IFN-β) 9 hours after infection corresponded to IBV pathogenicity. In summary, higher apoptosis and elevated levels of TLR3, MDA5 and IFN-β expression correlates to pathogenicity of IBVs in kidney (CEK cells) tissues.

To further analyse the differences in early host innate immune responses and apoptosis after IBV infection, we infected chicken tracheal organ cultures (TOCs) with IBV strains 885, QX and M41. We demonstrated that IBV strain M41 induced stronger innate immune response as indicated by up-regulations of TLR3, MDA5 and IFN-β and apoptosis than 885 or QX in TOCs. These observed effects suggest that higher apoptosis together with elevated levels of TLR3, MDA5 and IFN-β expression appears to be correlates to pathogenicity of IBVs in TOCs (respiratory tissue).

For corroborating theses *in vitro* results further work involved the differential immunopathogenesis in chickens infected with IBV strains 885, QX or M41. We confirmed that the histopathological changes, proinflammatory and innate immune gene response could be induced to varied degrees, depending on the IBV strain. Essentially, our results indicates that higher upregulation of expression of proinflammatory cytokines (such as IL-6 and IL-1β) and lipopolysaccharide-induced tumor necrosis factor-alpha factor (LITAF) is induced by M41 in trachea and 885 and QX in the kidney, which seem to mainly coincide with tracheal and renal histopathological lesions caused by these strains, respectively. In addition, elevated levels of TLR3, MDA5 and IFN-β expression correlated with lesion severity in IBV infected trachea and kidney tissues. Overall, this study reports striking differences in the activation of host responses by diffrerent pathogenic IBV strains.

Despite vaccination IBV 793B infections continue to be a major threat to the health of all types of chicken in many parts of the world. In this study, the immunopathogenesis of 793B serotype was comprehensively analysed after IBV infection either *in vitro* (CEK cells and TOCs) or *in vivo* (SPF chicks). In conclusion this study reports higher levels of apoptosis together with elevated TLR3, MDA5 and IFN-β expression *in vitro* which correlates to the pathogenicity of 793B in trachea and kidney *in vivo*. Furthermore, we observed that greater upregulation of proinflammatory cytokines (such as IL-6 and IL-1β) and LITAF is induced by 793B infection in trachea and kidney which appears to be partially implicated lesions caused by IBV. Our findings contribute to the underlying mechanisms of induction of host immune responses to IBV in SPF chicks *in vivo* as well as *in vitro.*

The project aimed to assess the mucosal, cellular and humoral immune responses induced by two different infectious bronchitis virus (IBV) vaccination regimes and their efficacy against challenge by a variant IBV Q1. Day-old broiler chicks were vaccinated with live H120 alone (Group I) or in combination with CR88 (Group II). Both groups were again vaccinated with CR88 at 14 days of age (doa). One group was kept as a control (Group III). All groups were challenged oculo-nasally with a virulent Q1 strain at 28 doa, and their protection was assessed. Both vaccinated groups gave excellent ciliary protection against the Q1, though group II's histopathology lesion scores and viral RNA loads in the trachea and kidney showed greater levels of protection compared to group I.

Declaration

All techniques and experiments performed and described in this thesis were undertaken by myself as a PhD student at the University of Liverpool between March 2013 and October 2015, unless otherwise acknowledged.

Neither this thesis nor any part of it has been submitted in support of an application of another degree or qualification of this or any other University or other institute of learning.

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Rajesh Chhabra

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Dedication

I dedicate my dissertation work to my family and friends. A special feeling of gratitude to my loving parents, wife Priti and my children Shreshtha, and Eva for their endless love.

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List of publications and conference attended

The following publication and presentation have resulted from the work described in this thesis:

Publications in peer-reviewed scientific journals

- R. Chhabra, J. Chantrey and K. Ganapathy. (2015). Immune responses to virulent and vaccine strains of infectious bronchitis viruses in chickens. Viral Immunology **28** (9): 478-488.
- Rajesh Chhabra, Anne Forrester, Stephane Lemiere, Faez Awad, Julian Chantrey, and Kannan Ganapathy. (2015). Mucosal, cellular and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and the protection conferred against infectious bronchitis virus Q1 strain. Clinical and Vaccine Immunology **22** (9): 1050-1059.
- Rajesh Chhabra, Suresh V Kuchipudi, Julian Chantrey and Kannan Ganapathy. (2015)**.** Pathogenicity and tissue tropism of infectious bronchitis virus correlates to elevated apoptosis and innate immune responses. Submitted to Virology **488** (1): 232-241.

Conference Publication

- R. Chhabra, S. V. Kuchipudi, J. Chantrey and K. Ganapathy (2014) Strain-dependent differences in the infection of primary chicken kidney cells by infectious bronchitis virus. In: Leirtz, M., Heffels-Redman, and Enderlein, D. ed (s) 8th *International symposium on avian corona-and pneumovirus and complicating pathogens. Rauischholzhausen, Germany*, pp 186-192.

- F. Awad, R. Chhabra, A. Forrester, M. Baylis, S. Lemiere, I. Capua, H. Hussein and K. Ganapathy (2014) Protection against middle east variant infectious bronchitis viruses: **Use of heterologous live vaccines in commercial broiler chicks**. In: Leirz, M., Heffels-Redman, and Enderlein, D. ed (s) 8th *International symposium on avian corona-and pneumovirus and complicating pathogens. Rauischholzhausen, Germany*, pp 317-324-192.

- R. Chhabra, S. V. Kuchipudi, J. Chantrey and K. Ganapathy (2015). Pathogenicity and tissue tropism of infectious bronchitis virus correlates to elevated apoptosis and innate immune responses. In: Munir Iqbal ed. First Global Alliance for Research on Avian Diseases (GARAD) conference, Strand Campus King's College London, 29 June to 1 July 2015, pp 60.

- R. Chhabra, A. Forrester, J. Chantrey and K. Ganapathy (2015). Cellular and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and the protection given against IBV Q1 strain. *XIXth Congress WVPA, Cape Town, Cape Town*, pp 151.

Presentations at national/ international seminar/meetings

- R. Chhabra, S. V. Kuchipudi, J. Chantrey and K. Ganapathy. Comparative study of in vitro cellular response to infection by different strains of infectious bronchitis virus. British Veterinary Poultry Association (BVPA) meeting. Harrogate, UK, 13th-14th March 2014 (Oral presentation).

- R. Chhabra, S. V. Kuchipudi, J. Chantrey and K. Ganapathy. Strain-dependent differences in the infection of primary chicken kidney cells by infectious bronchitis virus. 8th International symposium on avian corona-and pneumovirus and complicating pathogens and 2nd cost action meeting. Rauischholzhausen, Germany, 17th-20th, June 2014 (Oral presentation).
- R. Chhabra, S. V. Kuchipudi, J. Chantrey and K. Ganapathy. Pathogenicity and tissue tropism of infectious bronchitis virus correlates to elevated apoptosis and innate immune responses. First Global Alliance for Research on Avian Diseases (GARAD) conference, Strand Campus King's College London, 29 June to 1 July 2015 (Poster).
- R. Chhabra, A. Forrester, J. Chantrey and K. Ganapathy. Cellular and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and the protection given against IBV Q1 strain. WVPAC, Cape Town, South Africa, 7-11 September 2015 (Poster).

List of abbreviations

AF	Allantoic fluid
AIV	Avian influenza virus
ANOVA	Analysis of variance
Ark	Arkansas
bp	Base pair
CD	Ciliostatic dose
CD 4 or 8 (+)	Cluster of differentiation 4 or 8 (positive) T cells
cDNA	Complementary deoxyribonucleic acid
CMI	Cell mediated immunity
Conn	Connecticut
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
doa	Days of age
dpc	Days post challenge
dpi	Days post infection
E	Envelope
ECE	Embryonated chicken egg
EID_{50}	50% egg infectious doses
ELISA	Enzyme-linked immunosorbent assay
EXO	Exonuclease
F	Fusion
FBS	Fetal bovine serum

G	Glycoprotein
HA	Haemagglutinatinin
HEPES	2-hydroxy-ethyl-piperazine-N2-ethane sulphonic acid
HG	Harderian gland
HI	Haemagglutination inhibition
hpi	Hours post infection
HIR	Humoral immune response
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
ILTV	Infectious laryngotracheitis virus
Kb	kilobases
LITAF	Lipopolysaccharide-induced tumor necrosis factor (TNF)-α factor
M	Membrane
M2	Second matrix
Mab	Monoclonal antibodies
Mass	Massachusetts
MDA5	Melanoma differentiation associated protein 5
MEM	Minimum essential medium
MOI	Multiplicity of infection

mRNA	Messenger RNA
N	Nucleocapsid
NDV	Newcastle disease virus
ODs	Optical density
P	Phosphoprotein
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
qRT-PCR	Quantitative real time reverse transcription polymerase chain reaction
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
rpm	Revolution per minute
RT-PCR	Reverse transcription polymerase chain reaction
S	Spike
SAP	Shrimp alkaline phosphatase
SEM	Standard error of the mean
SH	Small hydrophobic
SPF	Specific pathogen free
SW	Sterile water
TBE	Tris-borate-EDTA
$TCID_{50}$	50% tissue culture infectious dose
TOC	Tracheal organ cultures

TLR	Toll-like receptor
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
VNT	Virus neutralization test

Chapter 1: Literature review

The content from this Chapter has been published (Appendix III) in peer-reviewed journal as below:

- R. Chhabra, J. Chantrey & K. Ganapathy. (2015). Immune responses to virulent and vaccine strains of infectious bronchitis viruses in chickens. Viral Immunology **28** (9): 478-488.

1.1 The disease

Infectious bronchitis (IB) is an acute and highly contagious viral disease causing severe economic losses for those involved in the chicken industry. It was first reported by Schalk and Hawn as a respiratory infection prominent in chickens ranging from 2 days to 3 weeks of age, with a high mortality rate and on post-mortem revealing congested respiratory pathways of the bird (Schalk & Hawn, 1931). Later a virus was established as the causative agent of this disease and named as infectious bronchitis virus (IBV) (Beach & Schalm, 1936). IBV has an incubation period of 24 to 48 hours and viral spread occurs rapidly among chickens in a flock by aerosol and mechanical means. The spread of infection between flocks on farms with in close proximity is also likely to occur by aerosol (Jackwood & de Wit, 2013). Since the virus has been reported to replicate in the chicken's intestine (Ambali & Jones, 1990), other routes of spread may include contact with faeces and fomites. Role of vertical transmission reported in case of IBV (Cook, 1971) is considered to be of little importance.

It is accepted that chickens are the most significant natural hosts of IBV, however, there are some reports of genetically very similar coronaviruses causing enteric disease in turkeys (Cavanagh *et al.*, 2001; Guy, 2000), and respiratory and kidney disease in pheasants (Cavanagh *et al.*, 2002; Gough *et al.*, 1996). Despite these IBV infection has also been detected in turkeys in Oman (Al-Shekaili *et al.*, 2015) and in apparently healthy ducks in China (Feng *et al.*, 2012).

IBV replicates in upper respiratory tissues, with infection of bronchi and severe disease in young animals. Some strains of IBV cause more systemic infections,

replicating in other tissues, including the kidney (causing nephritis), the oviduct (causing decreased egg production), and the gut (Ganapathy *et al.*, 2012). It has been reported that the virus can cause infertility in male chickens (Boltz *et al.*, 2007). While chickens of all ages are susceptible, very young chicks exhibit more severe respiratory signs and much higher mortality than older birds (Raj & Jones, 1997c). The severity of the clinical signs is influenced by several factors associated with the IBV (eg. strain, virulence and dosage), the host (eg. age, sex, type and immune status), the environment (eg. dust, ammonia and stress), management and biosecurity levels (Ganapathy, 2009). IBV respiratory infection causes characteristic, but not pathognomonic signs, such as gasping, coughing, tracheal rales and nasal discharge (Raj & Jones, 1997c). Puffy, inflamed eyes and swollen sinuses may occasionally be noticed and usually persist for 5 to 7 days and diminish within 10 to 14 days (Raj & Jones, 1997c). Infected chickens can also appear depressed with a remarkable decrease in weight gain and food consumption within 3 days post infection (dpi) (Otsuki *et al.*, 1990). Mortalities are usually low, possibly due to asphyxiation induced by the blocking of the lower trachea or bronchi by plugs of mucus (Raj & Jones, 1997c). In layers, IBV can cause egg production losses or quality decline with mild (Muneer *et al.*, 1986) or no respiratory signs (Cook & Huggins, 1986). However, the severity of decline in egg production quantity and quality is strain dependent (Jackwood & de Wit, 2013). In broilers, mostly the nephritic form of IBV occurs (Butcher *et al.*, 1989) and the stress of the disease reduces meat production by increasing the feed conversion ratio and reducing weight gain. These losses from production inefficiencies are usually of greater concern than losses from mortality. Mortality in broilers is economically significant

with peaks between 5 to 6 weeks, usually caused by secondary bacterial infection (Chandra, 1987). This association between IBV respiratory reactions and susceptibility to *E. coli* in chickens has been studied extensively (Avellaneda *et al.*, 1994; Cook & Huggins, 1986; Hopkins & Yoder, 1984; Smith *et al.*, 1985). The damage caused by IBV to the tracheal epithelium facilitates *E. coli* invasion and multiplication leading to 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

1.2.1 Genome and protein

IBV is a positive-sense single-stranded (ss) RNA virus of 120 to 160 nm in diameter and with typically large surface clubs of 20 nm, with heavily glycosylated spike projections (Figure 1.1). IBV belongs to Group 3 of the coronavirus genus within the Order *Nidovirales* and family *Coronaviridae* (Cavanagh, 2003). The IBV has a genome length of approximately 27.6 kilobases (kb) encoding both structural proteins; spike (S1 and S2), membrane (M), envelope (E), and nucleocapsid (N), and the non-structural proteins (Nsps) that are important for the proliferation or replication of the virus.

The S proteins are found on the surface of the viral envelope has two cleaved forms, S1 and S2 with molecular weights of 92k and 84k, respectively. The S1 subunit contained epitopes responsible for inducing neutralizing antibodies against IBV in chickens, while S2 is responsible for fusion of the virus with the host's cells (Belouzard *et al.*, 2012; Ignjatovic & Sapats, 2005). The S protein has also been associated with *in vitro* host cell specificity (Casais *et al.*, 2003; Casais *et al.*, 2001).

The M glycoprotein is embedded in 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), while E protein is a scant protein and contains highly hydrophobic transmembrane N-terminal and cytoplasmic C-terminal domains. Both proteins are important for virus particle formation during replication specifically, the E protein has the role of forming cation-selective ion channels within the host membrane to enhance viral morphogenesis, assembly and apoptosis (Corse & Machamer, 2003; Wilson *et al.*, 2004).

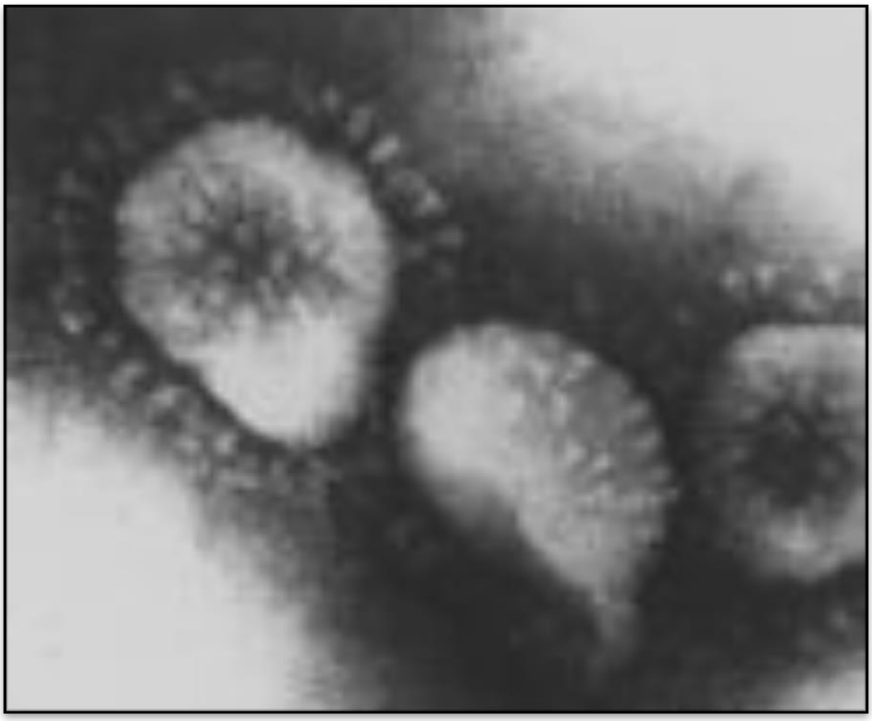


Figure 1.1. Electron micrograph of an IBV virion showing the club-shaped projections.

The N protein surrounds the single stranded positive sense RNA genome of IBV forming a structure termed as ribonucleoprotein (RNP) (Jackwood & de Wit, 2013; Lai & Cavanagh, 1997). The N protein is generally localised within the cytoplasm of the host cell, but as a strategy to control host and viral transcription of subgenomic RNA, may also migrate to the host cell nucleolus (Hiscox *et al.*, 2001). Coronavirus N-proteins including that of IBV also play a role in stimulation of cell-mediated immune responses against IBV 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-cells epitope peptides, which are conserved among avian coronaviruses, have also been mapped within the nucleocapsid N-terminal domain (Yu *et al.*, 2010).

IBV genome possesses two small nsp genes, 3 and 5, that express three (3a, 3b, and 3c) and two (5a and 5b) gene products, respectively. These mature and intermediate products are involved in the genomic and subgenomic RNA synthesis (Ng & Liu, 1998, 2000)

1.2.2 Virus replication

IBV replication starts with the binding of S protein of the virus to receptors present in the cell membrane (Belouzard, et al., 2012). Thereafter host-cell dependent proteolytic cleavage of the viral S protein and the fusion of the viral envelope with the plasma membrane are observed. It is not clear if the virus is internalized by either receptor-mediated endocytosis or by fusion with the host cell membrane. After the entry and uncoating of the viral particle into the host cell, the (+)ssRNA of the virus is used as a template by the host polymerase to synthesizes the viral RNA

polymerase directly. The viral RNA polymerase uses the (+)ssRNA as a template for the production of a full length negative-stranded (-)ssRNA, which is used as a template to produce genomic and subgenomic RNAs (Zhao *et al.*, 1993)**.** For the production of subgenomic length RNA, the viral polymerase transcribes short leader sequences found at the 3' end and allows for elongation either up to the first transcription-regulating sequence and stops synthesis, or to skip over this sequence to continue to elongate the nascent strand (Siddell & Snijder, 2008). The (-)ssRNA is used to synthesize the other proteins of the virus. The first step in virus assembly is the binding of N protein to viral RNA forming the helical nucleocapsid (Lai & Holmes., 2001), 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 a 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). The virions are then transported to the cell surface via the Golgi transport apparatus, and released into extracellular space by exocytosis.

1.2.3 Pathogenesis of IBV

IBV is primarily epitheliotropic and has wide tissue tropism including respiratory, urogenital and digestive system (Ganapathy, et al., 2012; Ignjatovic *et al.*, 2002). Although all IBV strains appear to infect the birds via the respiratory tract, later it finds favourite sites for further replication and persistence. The dissemination of IBV from the respiratory tract to other tissues is believed to occur through the process of viremia. Virus replication in ciliated epithelial and mucus-secreting cells

of the trachea (Nakamura *et al.*, 1991; Owen *et al.*, 1991; Yagyu & Ohta, 1990) and its isolation from trachea has been reported in many studies (Janse *et al.*, 1994; Lee *et al.*, 2002; Otsuki, et al., 1990). The persistence of virus in trachea is strain dependent. Infection by strain G in the upper respiratory tract shows the highest viral titres within 3 dpi and virus isolation was observed up to 14 dpi (Ambali & Jones, 1990). On the other hand IBV 793B was isolated from the trachea of infected specific pathogen free (SPF) chicks for up to 7 dpi (Raj & Jones, 1996a). Respiratory infection results in de-ciliation of the cells of the nares and trachea and inflamed lung tissue as well as thickened and cloudy air sacs along with the infiltration of mononuclear cells in the surrounding mucosal layers (Cavanagh, 2003). The tracheal damage in terms of ciliary activity is variable as it depends on the virulence of IBV strains involved (Otsuki, et al., 1990; Raj & Jones, 1997b). The severity of the ciliostasis caused by 793B strain proved to be the mild, while more severe effect was observed after M41 infection (Benyeda *et al.*, 2009). Respiratory signs are characterized by nasal discharge, sneezing, rales, watery eyes, and lethargy, combined with reduction in feed consumption and weight gain (Jackwood & de Wit, 2013). In addition, nephritis caused by viral replication in the kidneys is not uncommon during IBV outbreaks. The damage to the kidneys occurs in infections with the nephropathogenic strains of IBV, with the virus targeting the lower nephron of the kidney down to the collecting duct epithelial cells for replication (Cavanagh *et al.*, 1997). Furthermore, presence of high titres of the virus in the kidney does not correlate well with observed kidney disease, as Moroccan G strain replicated to similar titres in the kidney or trachea, though no gross kidney changes were observed (Ambali & Jones, 1990). These nephropathogenic strains produce

fewer respiratory signs (Ziegler *et al.*, 2002) and lesions (Glahn *et al.*, 1989) but can induce high mortality in younger birds (Cook *et al.*, 2001; Liu *et al.*, 2006).

IBV can replicate in the epithelium of the oviducts in young chicks (Crinion & Hofstad, 1972b) and in laying hens (Jones & Jordan, 1971). IBV infection in young pullets can result in permanent damage to the oviduct, resulting in a significant decrease in both egg quality and numbers, and the production of "false" layers who are unable to lay eggs due the formation of cysts within the oviducts (Boltz *et al.*, 2004; Jones & Ambali, 1987). The pathology of IBV infection in the male reproductive tract has not been well documented. IBV has been identified in the testes of cockerels, indicating a potential expansion in the tissue tropism for some strains of IBV, and the resulting danger of infertility in males if replication of IBV occurred in the spermatozoa producing cells of the testes (Boltz, et al., 2004).

As discussed earlier, IBV grows at many epithelial surfaces beyond the respiratory tract (Raj & Jones, 1997c) (reviewed by Raj & Jones, 1997). Although many alimentary tract tissues are susceptible to IBV, infection of enteric tissues usually does not manifest itself clinically (Cavanagh, 2003). IBV has also been isolated from various tissues such as lung, airsacs, oesophagus, proventriculus, duodenum, jejunum, liver, spleen, bursa of Fabricius, caecal tonsils, ileum, rectum, cloaca, semen and eggs (Ambali & Jones, 1990; Dolz *et al.*, 2012; Lucio & Fabricant, 1990; Otsuki, et al., 1990) and virus isolation from the Harderian gland (HG) has also been reported following experimental infection with live attenuated H120 (Toro *et al.*, 1996).

1.3 Serotype and strain variation

IBV strains have been classified into different serotypes based on the antigenicity of S protein. Traditionally serotyping has been performed using virus neutralization (VN) and haemagglutination inhibition (HI) tests (Toro *et al.*, 1987) however, cross-reactions may occur between serotypes especially when sera are collected from field samples (Jackwood & de Wit, 2013). In addition to this some laboratories also use ELISA with monoclonal antibodies directed against specific epitopes of the S1 protein (Jackwood & de Wit, 2013). In recent years genotype classification based on the S1 region of the spike gene has become the most widely used method for IBV typing (Cavanagh *et al.*, 1999; Jackwood *et al.*, 1992; Lee *et al.*, 2000), and replaced conventional HI and VN serotyping methods used for determining the identity of a field strain (Jackwood & de Wit, 2013). Genotype classification involves use of RT-PCR amplification of the S1 portion of the S gene. The cDNA is further confirmed by sequencing or RFLP analysis (Jackwood & de Wit, 2013). Despite these procedures being widely used in molecular epidemiology studies (De Wit, 2000), the correlation between genotype and serotypes of IBV strains has been contradictory. Strains of the same serotype tend to have S1 amino acid identity of ≥90% however, not all isolates from the same genotype belong to the same serotype (Cavanagh *et al.*, 1992).

After the initial description of IB in 1931, to date, a wide range of different IBV serotypes and genotypes have been reported around the world (Jackwood *et al.*, 1997; Sjaak de Wit *et al.*, 2011). The first isolate of IBV was the Beaudette strain (Beaudette & Hudson., 1937) and later, the M41 strain related to the Beaudette strain was isolated in the North Dakota, USA. Despite being first identified in USA,

the classical M41 serotype and the Dutch H120 serotype derived from a Dutch isolate of 1955 are the most widely used vaccine viruses (Sjaak de Wit, et al., 2011) Since then many different IBV variants have been isolated and characterised in different parts of the world.

In the USA, the most commonly found type of IBV is Arkansas (Ark) (Jackwood, 2012). Other commonly detected viruses in the USA are Connecticut **(**Conn) and Mass types (Jackwood *et al.*, 2005). In Europe the majority of the strains are related to 4/91 (793B, CR88), Italy 02 and D274 (Capua *et al.*, 1999; Cavanagh, et al., 1999). In recent years, IBV QX first identified in China (Wang Y, 1998), appears to have spread among poultry flocks in many countries (Bochkov *et al.*, 2006; Ducatez *et al.*, 2009; Terregino, Toffan, Beato*, et al.*, 2008; Worthington *et al.*, 2008) demonstrating a shift in geographical distribution and importance of the QX-like genotype. Another IBV genotype, the Q1, which was genetically and serologically distinct from IB classical strains, was reported in China from 1996 to 1998 (Yu *et al.*, 2001) and recently this Q1 IB strain has also been identified in Europe (Toffan *et al.*, 2011).

1.4 Variation in virulence

The evaluation of the pathogenicity of different IBV strains for the respiratory tissue has been found difficult to quantify (Cook *et al.*, 1986). Cubillos *et al.* (1991) has reported differences in virulence of IBV strains for the trachea based on the variability of the tracheal damage in terms of ciliary activity in unvaccinated chickens. It has been suggested that the tracheal damage in terms of ciliary activity is variable since it depends on the virulence of IBV strains for the trachea (Otsuki, et al., 1990; Raj & Jones, 1997b). The severity of the ciliostasis caused by 793B strain

proved to be mild, while the effect of M41 was more severe (Benyeda, et al., 2009). However, there have been conflicting reports on the difference in virulence among IBV strains using this *in vitro* system. Cook *et al.* (1976b) did not find marked differences when compared three strains of IBV on the basis of their effect on tracheal cilia. Raj and Jones (1996b) also reported little difference among several IBV strains using measurement of ciliary activity as a criterion for damage to the tracheal epithelium. However, Abd El Rahman *et al.* (2009) have revealed that the strains differed in their efficiency to infect the tracheal epithelium. Infection by Beaudette and QX resulted in a larger number of infected cells compared to Italy 02 and 4/91, when tracheal organ cultures (TOCs) were infected with the same amount of virus (Abd El Rahman *et al.*, 2009).

The severity of kidney lesions produced may vary depending on the IBV strains (Albassam *et al.*, 1986; Chandra, 1987). However, using a model to titrate kidney infectivity by intra-venous inoculation, no differences were found in the infectivity among Belgian field nephropathogenic IBV isolates (Lambrechts *et al.*, 1993).

Variations among IBV strains in terms of the severity of egg production decline in quantity and quality have also been reported (Jackwood & de Wit, 2013). In 1986, Cook and Huggins reported that some variant strains of IBV which had a marked effect on egg color caused only small decreases in egg production (Cook & Huggins, 1986). The Massachusetts and T strains were found to be virulent for the immature chicken oviduct while the Connecticut and Iowa 609 strains were not (Crinion & Hofstad, 1972a). An *in vitro* study using oviduct organ culture (OOC) system has compared the virulence of seven strains of IBV by calmodulin assay (Raj & Jones, 1996b). In this study, the isolate D207 was the most virulent while the enterotropic

G variant strain was the least virulent in terms of damage to oviduct epithelium (Raj & Jones, 1996b).

1.5 Immunity against IBV

1.5.1 Innate immunity

The innate immune response is the first line of defence and comprises a set of mechanisms, molecules and cells that often non-specifically target invasive pathogens as they enter the potential host. This can be achieved through physical barriers provided by skin and mucous membranes, soluble factors such as complements and acute phase proteins, anti-microbial peptides, and leukocyte subsets such as heterophils, macrophages, natural killer (NK) cells, mast cells, basophils and eosinophils.

One of the first reactive components of innate immunity against IBV infection is hyperplasia of goblet cells and alveolar mucous glands, leading to seromucous nasal discharge and catarrhal exudates in the trachea (Nakamura, et al., 1991). Five dpi, when a loss of cilia, epithelial degenerative changes and depletion of goblet cells and alveolar mucus glands have occurred, other immunological components become activated (Nakamura, et al., 1991).

1.5.1.1 Pattern recognition receptors (PRRs)

The PRRs are present on the cytoplasmic surfaces of immune cells, such as dendritic cells, macrophages, lymphocytes, and several non-immunological cells such as endothelial cells, mucosal cells, and fibroblasts. These cells rapidly recognize infectious agents through PRRs such as Toll-like receptors (TLR), retinoic acid-inducible gene 1 (RIG-I) like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD) like receptors (NLRs). TLRs are one of the primary

mediators of the innate immune system, recognising conserved structures in a broad range of pathogens. In chickens, the currently known TLRs are TLR-1 LA, TLR-1 LB, TLR-2A, TLR-2B, TLR-3, TLR-4, TLR-5, TLR-7, TLR-15 and TLR-21 (St Paul *et al.*, 2013). An increase in TLR-3 mRNA expression in tracheal epithelial cells after intranasal inoculation with IBV-M41 strain at 3 dpi has been reported (Wang *et al.*, 2006) and its function in viral immunology is well established (Le Goffic *et al.*, 2007).

In another study, TLR-1 LA, TLR-1 LB, TLR-2, TLR-3 and TLR-7 gene expression were all significantly upregulated in the tracheal epithelial cells of 3 weeks old chickens immunised with attenuated IBV-Massachusetts (IBV-Mass) by intranasal inoculation (Guo *et al.*, 2008). These studies have not shown whether host responses are elicited against IBV strains in lung tissue, though the lung is also a target organ for IBV. This was attempted in a more recent study (Kameka *et al.*, 2014), where 6-day-old SPF chickens were intra-tracheally infected with a Connecticut strain of IBV and thereafter, an up-regulation of TLR-3 and TLR-7 mRNA was identified in the trachea and lung. Conversely, down-regulation in the expression of TLR-3, along with IL-1β and IFN-γ, was also observed in the early phase (12 hpi) of viral replication. This early delay in induction of innate responses following infection was thought to be due to an increase in IBV genome load and worsening histopathological lesions in the trachea and lungs of IBV infected chickens. The chicken TLR-21 is a functional homologue of mammalian TLR-9 and after stimulation with deoxyoligonucleotides containing CpG motifs, it induces nuclear factor kappa-light-chain-enhancer of activated *B* cells (NFκ-B) production, leading to enhanced transcription of a number of cytokines (Brownlie *et al.*, 2009).

A decrease in viral load has been reported after treatment of 18-day-old embryos with deoxyoligonucleotides containing CpG motifs prior to inoculation with IBV (Dar *et al.*, 2009). Melanoma differentiation-associated protein 5 (MDA 5) expression levels were reported to be significantly increased in chicken kidney tissue after nephropathogenic IBV infection, suggesting a role for chicken MDA5 against IBV infection (Cong *et al.*, 2013). In a more recent study, it has been shown that *in vitro* virulent IBV infection leads to a significant induction of INFβ transcription through an MDA5-dependent activation of the IFN response (Kint *et al.*, 2015).

1.5.1.2 Heterophils

Activation of the innate immune response leads to the recruitment of phagocytic heterophils and macrophages. Heterophils form the polymorphonuclear cell population of the chicken and are the primary phagocytic cells that are first recruited to the infection site during the early stages of IBV induced inflammation in chickens, along with other lymphocytes in the HG and trachea of IBV-infected tissues (Nakamura, et al., 1991; Raj *et al.*, 1997; Toro, et al., 1996). The dramatic increase in heterophil numbers during IBV infection from 24-72 hpi has been reported in experimental studies by respiratory lavage analysis (Fulton *et al.*, 1993). Although heterophils lack the expression of major histocompatibility complex (MHC) class I and II molecules seen in other chicken innate immune cells, they are highly phagocytic and express most of the TLRs found in chickens (Brune, 1972). Heterophils have been proposed to be responsible for the destruction of IBV-infected cells during initial infection by phagocytosis and oxidative lysozomal enzyme release (Fulton *et al.*, 1997). This was supported by another study (Guo, et al., 2008), where an upregulation in the gene expression of heterophil cathepsin S

and bactericidal permeability-increasing protein (BPI) was detected in IBV-infected chicks. These antimicrobial cytotoxic molecules are released by heterophil degranulation to degrade and neutralize pathogens. The important role for heterophils in the chicken immune response is also evident as heterophil-depleted chickens infected with IBV showed more severe nasal exudation compared to controls (Raj, et al., 1997). In the tracheal epithelium however, heterophils did not reduce virus replication and worsened the severity of lesions (Raj, et al., 1997).

1.5.1.3 Macrophages

Macrophage and monocyte chemotaxis to the area of infection is regulated by the production of chemokines by the other innate immune leucocytes. Information on the functional roles of macrophages in response to IBV infection is scarce.

It is clear that virus-induced cytolytic necrosis accounts for many of the pathological changes that are observed in IBV infections (Dar *et al.*, 2005). However, an increase in the number of macrophages was found in spleens of chickens inoculated with IBV-M41 from 1 to 7 days post vaccination (dpv) (Dar, et al., 2005). Similarly, infiltration of macrophages into the tracheal and bronchial lumen of IBV-M41 infected chickens has also been reported when collecting respiratory lavage fluid between 24 and 96 hpi (Fulton, et al., 1993). A rapid influx of macrophages within hours post infection was also seen in the lung after individual or mixed avian pathogenic *E. coli* (APEC) and IBV infections (Matthijs *et al.*, 2009). These findings were confirmed in a more recent study (Kameka, et al., 2014), where macrophage numbers within the lungs and tracheas of chickens infected with Conn strain IBV were found significantly increased at 24 hpi compared to uninfected controls, suggesting that respiratory macrophages may

play an important role in limiting the replication of IBV within respiratory tissues. An upregulation in the gene expression of monocyte and macrophage signalling molecules has also been reported, including Spi-1/PU.1, glia maturation factor GMF-β and macrophage colony stimulating factor receptor M-CSFR in IBV-Mass infected chicks (Guo, et al., 2008). Spi-1 induces macrophage differentiation, GMF stimulates NF-κB, GM-CSF and CD4-/CD8+ cell differentiation and M-CSF is a key for macrophage linage development (Garceau *et al.*, 2010).

Unlike certain other avian viral respiratory infections, IBV was initially reported to have no effect on macrophage-mediated phagocytosis of serum opsonised *E. coli* (Naqi *et al.*, 2001). Macrophages isolated from peripheral blood or the respiratory tracts of chickens infected with IBV-M41 showed effective phagocytic activity or bactericidal function for *E. coli in vitro* (Naqi, et al., 2001). However, IBV was later shown to lower the bactericidal activity of peripheral blood mononuclear cells (PBMCs) and splenocytes, though their phagocytic capacity and recruitment remained unaffected (Ariaans *et al.*, 2008).

1.5.1.4 Dendritic cells

Dendritic cells (DCs) are members of the mononuclear phagocytic system. Innate activation of a variety of these cell subsets can increase their capacity to interact with T cells , the most important of these being the DC subsets (Helle R. Juul-Madsen *et al.*, 2014). DCs play an important role in the initiation of the adaptive immune response, being the only cells that can activate naive T cell subsets by virtue of their high levels of MHC molecules and co-stimulatory activity (e.g., expressing CD80/86) (Helle R. Juul-Madsen, et al., 2014). Still little is known about the function and migration of chicken DCs (Wu *et al.*, 2010). In recent years, with

the emergence of new chicken DCs markers, it has become increasingly possible to culture chicken DCs *in vitro* (Fu *et al.*, 2014; Wu, et al., 2010). However, currently no publications are available on the nature of IBV interactions with chicken DCs. Hence, further characterization and studies on this cell may yield important immunological mechanisms conferring protection against virulent or vaccine strains of IBV.

1.5.1.5 Natural killer cells

Natural killer (NK) cells are cytotoxic lymphocytes that play a key role in the early defence against viral infections. As opposed to in mammals, NK cells are found in very low numbers in the spleen or peripheral blood of birds (Göbel, 2001). The role of NK cells in IBV infection has not been studied extensively. It was initially reported that an IBV vaccine designated as the Holland strain, Mass serotype, had caused no alterations in NK cell activity (Wakenell *et al.*, 1995). However, it was later shown that infection of chickens with IBV-M41 induced rapid NK cell activation in the lung and blood (Vervelde *et al.*, 2013). Five week old chickens receiving IBV-M41 via intratracheal and intranasal routes showed an increase of $CD107^{+}CD3^{-}$ NK cells in the lungs only on 1 dpi, whereas the change of NK cells in PBMC levels was biphasic with an increase on 1 and 4 dpi but not 2 dpi.

1.5.1.6 Acute phase proteins

In general, inflammatory cytokines and chemokines stimulate the production of acute phase proteins (APP) in chickens, including αI acid-glycoprotein (AGP), mannose binding lectin (MBL), transferrin-ovotransferrin (OVT), fibrinogen and C-reactive protein. AGP has various biological activities, including immunomodulation of the inflammatory response, and its concentration has been

recorded to increase during IBV infection (Asif *et al.*, 2007). MBL plays a role in the innate immunity against IBV through an acute phase response, whereby it is able to activate complement and inhibit the propagation of the virus in the trachea (Juul-Madsen *et al.*, 2007). MBL has also been found to be associated with increased disease severity after IBV infection, as lower levels of MBL in serum lead to increased replication of IBV in chickens (Juul-Madsen *et al.*, 2011). Another recent study by Kjaerup *et al.* (2014) has shown that MBL has the capability to bind to IBV *in vitro* and is involved in the immune response to IBV vaccination. In their study, two inbred lines (L10H and L10L) selected on the basis of high or low MBL serum concentrations, respectively, were vaccinated against IBV with and without the addition of the MBL ligands mannan, chitosan and fructooligosaccharide (FOS). The addition of MBL ligands to the IBV vaccine, especially FOS, enhanced the production of IBV-specific IgG antibody in L10H chickens, but not L10L chickens after the second vaccination, suggesting that MBL is involved in the immune response to IBV vaccination. Elevated levels of OVT in serum, which is a moderate APP, have also been documented after challenge with IBV (Xie *et al.*, 2002).

1.5.1.7 Complement system

The complement system, as a component of innate immunity, is immediately ready to target and eliminate virus particles and to interact with the surface of virus infected cells (Baelmans *et al.*, 2006). The trachea of IBV-Mass infected chickens demonstrated an increase in the gene expression of C1q, C1s, anaphylatoxin C3a receptor and complement C4, and a down-regulation in factor H (an inhibitor of the complement system) (Guo, et al., 2008). C1q and C4b mediate opsonisation and the

activation of the classical pathway of the complement system whilst C3a and C4a elicit local inflammatory responses to limit infection (Guo, et al., 2008).

1.5.1.8 Cytokines

Cytokines are crucial regulators of the immune system (both innate and adaptive), and bind to specific cell surface receptors to initiate cascades of intracellular signalling for specific cell functions (Kruth, 1998). The infection of chicken with attenuated IBV-Mass elicited the gene expression of IL-1β (10.8-,5.5-, 2.1-, and 2.5-fold for 1, 3, 5, and 21 dpi), IL-10R2 (6.9-, 2.4-, and 2.3-fold for 1, 3, and 12 dpi), and common cytokine receptor g (increased 2.4-, 4.0-, 4.4-, and 2.9-fold from 1 to 8 dpi) to bind IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21 in the tracheal tissue (Guo, et al., 2008). IBV infection has previously been found to induce IFNs in the trachea, lung and at lower levels in the plasma, kidney, liver and spleen (Otsuki *et al.*, 1987). The role of the proinflammatory cytokine, IL-6, has been investigated for its contribution to nephritis in the two genetic line of chicken regarding the susceptible S-line and a more disease-resilient HWL line infected with T strain of IBV (Asif, et al., 2007). Although IL-6 mRNA levels were elevated in the kidneys of both bird lines at 4 dpi, these levels were 20 times higher in S line chickens than HWL chickens. Furthermore, serum IL-6 levels were found to be three times higher in S-line chickens when compared to HWL chickens after IBV infection, suggesting that IL-6 may play a role in IBV-induced nephritis. Differential immune responses of chickens using two IBVs with different genotypes, KIIa and ChVI have also been reported (Jang *et al.*, 2013). In chickens infected with KIIa genotype, at 7 dpi in the trachea and 9 dpi in the kidney, simultaneous peaks occurred in the number of virus copies and the upregulation of mRNA levels of pro-inflammatory cytokines (IL-

6 and IL-1β) and lipopolysaccharide-induced tumor necrosis factor (TNF)-α factor (LITAF). This appeared to contribute to the scale of pathophysiological effect in the chickens. Alternatively, chickens infected with ChVI genotype showed comparatively mild upregulation in pro-inflammatory cytokines mRNA expression. In a recent report, after infection with IBV-M41 strain, an early (3 dpi) upregulation of proinflammatory cytokines IL-6 and IL-1β was reported. This coincided with the highest viral loads and microscopic tracheal lesions, indicating a role for both of these cytokines with high virus loads and the development of tracheal lesions (Okino *et al.*, 2014).

In another study, a disorder was hypothesised in the expression of eggshell components in laying hens after IBV infection, linked with the expression of these proinflammatory cytokines (Nii *et al.*, 2014). A significant down-regulation in the relative expression of IFN-γ and IL-1β mRNA has also been noted within the trachea during the initial phase of Conn strain IBV infection when compared to uninfected controls. Conversely, IL-1β mRNA showed a sharp increase in expression as the IBV infection progressed (Kameka, et al., 2014). The transcriptomics study of the host kidney, in response to IBV infection, revealed that viral infection contributed to differential expression of 1777 genes, of which 876 were up-regulated and 901 down-regulated compared to those of control chickens, and103 were associated with immune and inflammatory responses (Cong, et al., 2013). In this study, increased expression of IL-6, IL-18, IL-10RA, IL-17RA, CCL4, CCL20, CCL17, and CCL19 was found after IBV infection. However, chemokine (C-X-C motif) ligand 12 expressions were found to be decreased.

The studies involving IBV sensitivity to chicken interferon (ChIFN) have shown conflicting results. Initially, Holmes and Darbyshire (1978) reported that none of the six strains of IBV examined were sensitive to ChIFN as tested in cultures of chick embryo tracheal rings. In contrast, Otsuki *et al.* (1979) reported that all 10 strains of IBV tested were sensitive to the action of IFN in chicken kidney cells (CKC). Later, type I interferon, IFN-α, was reported to inhibit IBV (Beaudette or Gray strains) replication both *in vitro* (CKC) and *in vivo* (Pei *et al.*, 2001). Intravenous or oral administration of IFN-α prevented IBV-related respiratory clinical signs by delaying the onset of the disease and decreasing the severity of illness (Pei, et al., 2001). Moreover, IBV stimulated chicken IFN-γ production in leukocytes of both infected birds and uninfected birds (Ariaans *et al.*, 2009). This stimulation was reduced when IBV was inactivated, but IFN-γ production still remained elevated when compared to unstimulated cells (Ariaans, et al., 2009). Recently, an increased expression of IFN-γ was reported in the lungs of IBV-M41 infected birds at 2 to 4 dpv and in the PBMCs within 1 dpv (Vervelde, et al., 2013). In a 2014 study, IFN-γ was upregulated in the trachea after infection with IBV-M41 which implicated viral infection in the tracheal pathology of non-immune challenged chickens (Okino, et al., 2014) .

1.5.1.9 Chemokines and other factors for immune cell trafficking

Chemokines orchestrate the migration of cells during immune surveillance. Attenuated IBV-Mass stimulated the gene expression of CXCR4 (2.1-, 2.1-, 3.0-, 2.2-fold for 1, 3, 5, and 12 dpi), CCR6 (2.0-, 2.5-,2.2-fold for 1, 3, and 5 dpi), chemokine-like receptor 1/CHEMR23 (3.0- and 2.9-fold for 1 and 3 dpi) in chicken trachea (Guo, et al., 2008). In addition, integrin β2 (CD18) was also upregulated in

the tracheas of chickens following attenuated IBV-Mass infection, which indicates the activation of leukocyte chemotaxis and therefore chemokine production (Guo, et al., 2008). Matrix metalloproteinase (MMP) levels were also increased implicating the migration of immune cells, possibly T cells, in the trachea of IBV infected chicks (Guo, et al., 2008).

Most recently an association of CpG-ODN induced changes in cytokine/chemokine gene expression has been found to coincide with suppression of IBV replication in chicken lungs (Dar *et al.*, 2014). The data showed that significant differential suppression of IL-6 gene expression and up-regulation of IFN-γ, IL-8 (CXCLi2) and MIP-1β genes was associated with inhibition of IBV replication in lung tissue from embryos which were pre-treated with CpG ODN.

1.5.1.10 IBV and apoptosis

Apoptosis is a highly controlled mode of cell death characterized by cell shrinkage, plasma membrane blebbing and nuclear fragmentation (Taylor *et al.*, 2008). The induction of apoptosis represents one of the major components of the host antiviral responses containing virus replication by rapid induction of cell death following infection of target cells. Nonetheless, viruses establish intricate and complex interactions with the host to regulate apoptosis to ensure a successful replication cycle that allows the production and spread of virus progeny to neighbouring cells (Hay & Kannourakis, 2002). Studies involving IBV Beaudette showed that it induced cell cycle arrest and apoptosis in infected Vero cells (Li *et al.*, 2007; Liu *et al.*, 2001). It has been demonstrated that IBV induces apoptosis in late stage of infection and identified proapoptotic (e.g., Bcl-2 associate X protein) and anti-apoptotic proteins (e.g., Bcl-2 and Bcl-XL) from the B-cell lymphoma 2 **(**Bcl-

2) family that modulate IBV-induced apoptosis (Li, et al., 2007; Liu, et al., 2001; Zhong, Liao*, et al.*, 2012; Zhong, Tan*, et al.*, 2012). In a recent study involving the role of unfolded-protein response (UPR) sensor IRE1α in IBV Beaudette-induced apoptosis, it has been found that IBV induces ER stress in infected cells and activated the IRE1α-XBP1 pathway at late stage of infection (Fung *et al.*, 2014). Moreover, Cong *et al.* (2013) have concluded in their study that apoptosis is a nonspecific defence mechanism against IBV infection through abortion of viral multiplication by premature lysis of infected cells.

1.5.2 Adaptive Immunity

Augmenting innate immune mechanisms, adaptive immunity allows the activation of antigen-specific effector mechanisms including B-cells, T cells, macrophages and the production of leucocytic memory cells which play a significant role in antiviral immunity against IBV.

1.5.2.1 Humoral immunity

Research of the humoral immune response to IBV has been extensively studied since the first detection of the virus. Upon immunostimulation, B-cells differentiate into plasma cells and secrete antibodies either in the presence or absence of T-helper cells. Chickens develop a humoral response to IBV infection, which can be continually measured in serum by ELISA, HI or VN serological tests (Raj & Jones, 1997c). The measurement of different classes of IBV-specific antibodies in particular tissues, after an IBV infection, has also gained importance.

1.5.2.1.1 Antibody kinetics

IgG (also known as IgY in chickens) is the major circulating immunoglobulin, and the kinetics of the IgG response to IBV is very different to that of the IgM response.

Anti-IBV IgG can be detected in serum within 4 dpi, peaking at about 21 dpi with the high titre possibly remaining for many weeks (Mockett & Darbyshire, 1981). Thus, the primary IgG response lasts much longer than the IgM response. After two vaccinations, serum IgG levels were much higher than those observed in the primary response but follow a similar pattern as observed after the primary vaccination (Mockett & Darbyshire, 1981). Moreover, *in vitro* stimulation of chicken PBMCs and splenocytes with IBV activates memory B cells to secrete antibodies at 21 dpi (Pei & Collisson, 2005). Memory B cells secreting IBV antibody (IgG) were detected by ELISPOT assays in both PBMCs and splenocytes collected from chicks after 3 to 7 dpi infected with IBV Gray strain (Pei & Collisson, 2005). A study by Raj and Jones (1996a) showed that IgG antibody content was highest in tears at 7 dpi and was still detectable at 23 dpi. However, significant levels of IgG antibody were also present in oviduct washes at 7 and 23 dpi.

IBV IgA antibodies have been detected in the lamina propria, tracheal washes and between epithelial cells in the trachea of IBV infected chickens (Joiner *et al.*, 2007). IBV-specific IgA antibodies have also been demonstrated in lachrymal fluid (tears), which correlated with resistance to IBV reinfection (Davelaar *et al.*, 1982; Gelb *et al.*, 1998). IBV-specific IgA in lachrymal fluid was initially detected 10 days after vaccination with live attenuated Ark DPI-type IBV vaccine. However, no further significant increase was noticed for IgA after subsequent challenges with Ark-IBV isolate AL/4614/98, clarifying the probable role of lachrymal fluid neutralizing antibodies at the time of challenge. This stimulation of the IgA antibody response upon challenge acts to reduce the potency of IBV infection (Joiner, et al., 2007). Raj and Jones (1996a) reported that tears showed the highest IgA antibody

concentration on 7 dpi but this decreased to an insignificant level by 17 dpi. In addition, 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). Alternatively, after ocular vaccination with a low passage, mildly pathogenic Ark-type IBV isolate, IBV-specific IgA secreting cells increased at 9 dpv in the HG. Moreover, IBV-specific IgA was found in cecal tonsils at 14 dpv, representing a delay in the IgA response at this site compared to that in the HG (van Ginkel *et al.*, 2008). In a recent study, it has been demonstrated that ocular vaccination with live attenuated Ark IBV vaccine induced higher IgA antibodies (in tears and plasma) in the primary IBV response, while the memory response is dominated by IgG antibodies. Therefore, lower mucosal IgA antibody levels are observed upon secondary exposure to IBV, which may contribute to increased susceptibility of host epithelial cells to reinfection by IBV and the persistence of the Ark serotype (Orr-Burks *et al.*, 2014).

1.5.2.1.2 Role of antibodies in protection

Maternally-derived antibodies can provide a short-lived resistance to IBV (Cook *et al.*, 1991; Darbyshire & Peters, 1985). Newly hatched chicks would therefore require to develop specific humoral responses to counter IBV infections. However, the precise role of antibody in the control of IBV infections remains controversial. Several studies have shown that circulating antibody titres do not highly correlate with protection from IBV infection. A lack of correlation between infectivity, serological response and challenge with IBV vaccine was first reported in 1965 (Raggi & Lee, 1965). Later in 1979, no correlation was found between HI antibody titres and susceptibility to challenge, as measured by re-isolation of virus from the

trachea (Gough & Alexander, 1979). Nevertheless, other studies have demonstrated that humoral immunity plays an important role in disease recovery and virus clearance. Cook and co-workers (Cook, et al., 1991) have reported that following IBV infection, bursectomised chicks showed more severe and longer lasting illness than intact chicks. The viral titres in tissues were also higher and lasted longer in bursectomised chicks compared to normal chicks though no difference in mortality was observed (Cook, et al., 1991). Similar earlier results were also reported using cyclophosphamide-treated chickens, which showed severe clinical signs and more severe histopathological renal lesions due to the prolonged persistence of IBV compared to controls (Chandra, 1988). It has also been reported that high titres of humoral antibodies correlate well with the absence of virus re-isolation from the kidneys and genital tract (Gough *et al.*, 1977; Macdonald *et al.*, 1981; Yachida *et al.*, 1985), and protection against a drop in egg production (Box *et al.*, 1988). Later, IBV-specific antibodies were suggested to be involved in limiting IBV spread by viraemia from the trachea to other susceptible organs including the kidneys and oviduct (Raj & Jones, 1997c).

In general, serum 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). Thus, antibodies at mucosal surfaces could contribute to this protection as IBV enters through this site and initially replicates in the HG and trachea. However, the role local antibodies play in preventing the re-infection is not clear. Some studies have reported that local antibodies plays a role in protecting the respiratory tract principally in the prevention of re-infections (Hawkes *et al.*, 1983; Holmes, 1973) and that the HG

contributes to the protective local immunity (Hawkes, et al., 1983; Toro & Fernandez, 1994; van Ginkel, et al., 2008). Alternatively, Gelb *et al.* (1998) found that some chickens with high tear IBV antibody titres were susceptible to IBV and that some chickens with low tear titres were protected, suggesting that mechanisms other than antibody mediated immunity in tears are important in viral clearance following infection.

Studies on isotype of antibodies involved in protection showed that after vaccination of broilers with IBV vaccine (H120 and D274 combination), the groups with 50% positive sera in IBV specific IgM at 10 days post vaccination had better protection against IBV-M41challenge (De Wit *et al.*, 2010). However, most groups of broilers with low levels of IBV specific IgM had a low or moderate level of protection against IBV-M41 challenge (De Wit, et al., 2010). It has been demonstrated that IBV-specific IgG responses were less protective against IBV than IBV-specific IgA antibodies found in tears (Toro & Fernandez, 1994). Furthermore, IBV-specific IgA antibodies were first detected in tears and later in serum, which suggests that IgA is important in neutralizing IBV at mucosal surfaces and is thought to play a role in the control of IBV locally (Davelaar, et al., 1982). In a study involving transcriptome analysis of tracheal samples from chickens ocularly vaccinated with attenuated IBV-Mass, a marked decreased was reported in expression of IgA at 8 and 12 dpi. Conversely, an up to 7-fold increase in the expression of IgG at 5 and 8 dpi was demonstrated, suggesting that IgA might not be important in protection against IBV infection of the upper respiratory tract whereas locally produced IgG, after a secondary immunization, provided effective protection against IBV by neutralizing this virus (Guo, et al., 2008). This was also

supported by a study that showed a significant correlation between the levels of lachrymal anti-IBV IgG at 5 dpi in chickens vaccinated with the full dose of H120 and tracheal protection against homologous IBV challenge (Okino *et al.*, 2013). In addition, a recent study also corroborates this hypothesis which reported that secondary antibody response at mucosal sites (lachrymal secretion) induced by attenuated IBV vaccines are dominated by an early and high production of anti-IBV IgG in re-immunized chickens (Orr-Burks, et al., 2014).

1.5.2.2 Cellular Immunity

Our understanding of immunity against IBV is deficient, especially the roles of cell-mediated responses (Cook *et al.*, 2012). Several studies examined the CTL responses in chickens to IBV infections and its correlation with early decreases in infection and clinical signs (Pei, et al., 2001; Seo & Collisson, 1997). CTL activity was dependent on the MHC and lysis was mediated by $CD8^{+}CD4^{-}$ cells. In addition, the passive transfer of IBV-infection induced αβ T lymphocytes bearing $CD8^{+}$ antigens protected naive chicks challenged with IBV (Seo *et al.*, 2000).

Antigen specific CTL activity was detected at 3 dpi and reached its peak level at 10 dpi in chickens infected with nephropathogenic Gray strain of IBV (Seo & Collisson, 1997). Throughout this period, acute IBV infection was contained and detectable IBV was neutralized. Viral titres were decreased in the lungs and kidneys of IBV infected chickens, and this was followed by a decline in CTL activity 10 dpi (Seo & Collisson, 1997). Such memory T cells persisted up to at least 10 weeks in blood post infection with IBV (Pei *et al.*, 2003). However, the duration of these memory T cells in tissues such as lungs, kidneys, spleens and trachea needs to be further examined in chickens.

During the course of experimental viral infection, Kotani *et al.* (2000) assessed the immunophenotype of mononuclear cells in the tracheal mucosa. The CTLs were found to be significantly increased in the tracheal mucosa after 3 or 4 dpi, peaking at 5 dpi and then decreasing to baseline levels by 14 dpi. So these infiltrating CTLs at the trachea mucosa were proposed to be involved in the clearance of IBV at this site, in an early phase of infection.

Effector $CD8^+$ T cells were shown to be critical in containing acute IBV infection (Collisson, et al., 2000). Adoptive transfer of immune T cells to chicks, prior to infection, demonstrated that IBV primed $CD8^+$, αβ T lymphocytes could protect chicks from acute infection (Collisson, et al., 2000; Pei, et al., 2003). The kinetics of viral load in the lungs and kidneys of IBV (Gray strain) infected chicks correlated with the level of IBV-specific CTL activity of effector cells isolated from spleen of the same infected chicks (Collisson, et al., 2000). Such IBV-specific CTL activity was dependent on the S and N proteins of IBV (Collisson, et al., 2000). Furthermore, the CD8+ T cell response in the blood and spleen occurred prior to the serum IgG antibody response to IBV (Liu *et al.*, 2012) supporting the hypothesis that the CTL response correlated with decreased viral load and an improved clinical response. *In vitro* studies have shown that CTL activity also induced IBV (Gray strain)-infected cell lysis. This lysis was dependent on the concentration of effector T lymphocytes, mostly $CD8^+$, with lower effect from $CD4^+$ cells (Collisson, et al., 2000). $CD4^+$ lymphocytes' minimal effect was suggested to be possibly due to either $CD4^-/CD8^+$ or $CD4^+/CD8^-$ (Collisson, et al., 2000). In another study, donor $CD8^+$ memory T cells protected recipient chicks from acute IBV infection for the first 4 dpi, and showed mild clinical illness at 5 dpi (Pei, et al., 2003). In addition, adoptive transfer of $CD4^+$

T cells did not appear to be important in initially containing IBV infection in chickens (Seo, et al., 2000). However, the low levels of protection in IBV infected chicks, that received $CD4^+$ T cells, was suggested to be because of contamination by CD8+ T cells (Pei, et al., 2003).

In 1991, the first IBV-specific T cell epitope in the IBV nucleoprotein (IBV N_{71-78}) from an IBV H52 strain was described (Boots *et al.*, 1991). This study revealed that only the S1 and N proteins of IBV generated cytotoxic T cell responses, but not the M protein of IBV. Later, the whole N protein and its carboxy-terminal region were reported to induce a CTL response, but not its amino terminal region (Seo, et al., 1997). This was further supported by another study (Guo *et al.*, 2010) wherein, increased numbers of CD4+ and CD8+ T cells in the PBMCs were found after vaccination of chickens with a DNA vaccine containing the sequence of N protein. In addition, immunization of chickens with a DNA vector expressing GM-CSF and S1 subunit of the spike protein, resulted in high antibody levels in serum, lymphocyte proliferation (including CD4+ and CD8+ T cells in PBMCs), and reduced the severity of clinical signs and mortality rate after challenge (Tan *et al.*, 2009).

Cytotoxic enzymes, granzymes and perforin, which are secreted by cytotoxic cells such as CTLs, γδ T cells and NK cells etc., play an important role in the cytotoxic activity induced following exposure to IBV (Vervelde, et al., 2013; Wang, et al., 2006). Transcriptome analysis of tracheal cells at 3 dpi with IBV-Mass revealed induction of genes involved in cytotoxicity such as Granzyme-A precursor, Fas, CD3 δ and γ chain, MHC II, and TLR-2. This expression pattern could be due to cytotoxic T cell responses as suggested by the authors (Wang, et al., 2006). More recently, after infection with IBV-M41, a late (7 dpi) induction of CD8αα and Granzyme

homolog A mRNA has been reported to be associated with highest scores of viral load and microscopic lesions in trachea, suggesting a role of both these cytotoxic enzymes and virus load on the development of tracheal lesions (Okino, et al., 2014). In addition, one study showed that the levels of expression of these genes evaluated at mucosal sites correlated significantly with the parameters of tracheal pathologic changes (viral load, histopathology and ciliostatsis) against homologous IBV challenge in a vaccine dose-dependent manner (Okino, et al., 2013).

1.5.3 Maternal derived antibodies (passive immunity)

Passive immunity was initially suggested protect young chicks from the development of abnormal oviducts when challenged with virulent field strains of IBV (Broadfoot *et al.*, 1954). IgG antibodies were detected in serum and respiratory mucus of newly hatched chicks that passed from vaccinated hens, via the yolk, to the progeny (Hawkes, et al., 1983).

In another study, chicks hatched with high levels of maternally-derived antibodies were also found to have excellent protection (>95%) against IBV challenge (IBV-Mass strain) at 1 day of age but not at 7 days (>30%) (Mondal & Naqi, 2001). This protection significantly correlated with levels of local respiratory antibody and not with serum antibody. Neither group of chicks produced IBV-specific antibodies when vaccinated with live IBV-Mass vaccine at 1 day of age by the intraocular route (Mondal & Naqi, 2001). Live vaccination of 1-day-old chicks induced a rapid decline in maternally derived antibodies due to the binding and partial neutralization of vaccine virus by these antibodies (Mondal & Naqi, 2001). Moreover, de Wit and colleagues recently challenged 6 or 10 day old chicks and reported that maternally derived IBV (D388 serotype, QX genotype) neutralizing antibodies provide partial

protection against tracheal damage and a high protection against viral replication in the chicks' kidney (de Wit *et al.*, 2011). Such protection was achieved in SPF layer breeders with a combination of heterologous live and inactivated IBV vaccines to boost their production of IBV maternally-derived antibodies (de Wit, et al., 2011).

1.6 Control of IBV

To control IBV infection in the field, IB vaccination programmes in broilers and breeders include application of both live attenuated and killed or inactivated vaccines (Jackwood & de Wit, 2013). Live attenuated IB vaccines are the first generation IBV vaccines which have been attenuated by successive passage in ECE (Klieve & Cumming, 1988). Experimental administration of live vaccine can be performed individually by oculonasal (Cook JKA, 1999) or intratracheal routes (Yachida *et al.*, 1986). However, in the field live vaccines are usually applied via drinking water, by coarse spray and aerosols at 1 day or within the first week of age (Jackwood & de Wit, 2013). Though these mass administration methods are popular because of convenience, problems in attaining uniform vaccine application can occur, and the aerosol method may cause more severe respiratory reactions. As the duration of immunity following live vaccine administration is short, a booster vaccination is carried out with the same strain or combination of other strains, 2-3 weeks after prime vaccination (Cavanagh, 2003). This combination of two serologically different vaccines (e.g. Ma5 and 4/91) has been shown to give wide immunity against many heterologous strains (Cook, et al., 2001; Cook JKA, 1999; Terregino, Toffan, Serena Beato*, et al.*, 2008). In addition, this vaccination strategy was found to be more effective than revaccination with a vaccine of the same serotype as the first vaccine (Cook JKA, 1999).

Most commonly live vaccines are derived from virulent strains such as M41 serotype and the Dutch H52 and H120 strains (Jackwood & de Wit, 2013; Lee *et al.*, 2010). However, in order to obtain optimal protection, variation may exist among countries on the type of IBV vaccine strain approved for use depending on the prevalent serotypes. For example, in the USA, M41, H120, Conn, JMK, Ark and Cal99 derived vaccines are frequently used for vaccination programme (Alvarado *et al.*, 2003; Gelb *et al.*, 1989; Martin *et al.*, 2007). In Australia, the B and C strains are being used. In Europe, vaccine strains include M41, 793B (4/91 and CR88) and D274 (Cook JKA, 1999; de Wit, et al., 2011; Terregino, Toffan, Serena Beato, et al., 2008). Despite these, some strains with regional or local impact have been used in different parts of the world. In some counties in Asia, apart from M41, 793B (4/91 and CR88) and D274, vaccines based on the local strains have been used widely (Lin *et al.*, 2005).

Inactivated or killed vaccines have been used either alone or in combination with live attenuated IBV vaccines to obtain long-lasting immunity to protect breeders and layers prior to the onset of egg production (Cavanagh, 2003; Finney *et al.*, 1990). These vaccines usually are administered by the intramuscular or subcutaneous route to layers and breeders at 13 to 18 weeks of age for induction of high levels of humoral antibodies, which can protect the oviduct of the birds against circulating IBV and avoids drops in egg production or quality. In the poultry industry the practice of vaccinating young females with live vaccines two or more times, followed by one dose of killed vaccine before the birds come into lay, is very common. However, in comparison to live vaccines, killed vaccines alone induce short-lasting immune response which is characterized by 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 the proper priming with live vaccines (Cavanagh, 2003; Jackwood & de Wit, 2013).

In the recent past with the advancements in molecular biology techniques, novel approaches such as sub-unit vaccines, plasmid DNA vaccines and vector-based vaccines along with reverse genetics vaccines have been tried. The experimental recombinant vector vaccines most commonly containing IBV-S1-glycoprotein have been developed against IBV. These vaccines were shown to induce significant increase in the immune response and protect against tracheal lesions following homologous and heterologous challenge 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 that include issue of pre-existing immunity or maternally derived immunity that interferes with the live vector itself and reduces the uptake of the antigen by the antigen presenting cells (Faulkner *et al.*, 2013).

However, oral immunization of mice with adenovirus vector was shown to avoid neutralization of the vector by maternally derived antibody (Xiang *et al.*, 2003). Interestingly, such adenovirus vector vaccines have been shown to be promising for use in poultry oral vaccines as they have several advantages including ease of application.

A DNA vaccine based on the S1-gene of Arkansas IBV serotype was developed (Kapczynski *et al.*, 2003). Immunization via the *in ovo* route with this vaccine, followed by application of a live attenuated vaccine at 2-week intervals, resulted in

a significant immune response and 100% protection against clinical disease (Kapczynski, et al., 2003). Enhancement of a vaccine-induced immune response was achieved by co-administration of a DNA vaccine encoding for IBV N or S1-glycoprotein genes with IL-2 (Tang *et al.*, 2008) or chicken granulocyte-macrophage GM-CSF, respectively (Tan, et al., 2009).

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 from pathogenic M41 and 4/91 strains, respectively (Armesto *et al.*, 2011). Use of this recombinant vaccine was shown to result in protective immune responses against the IBV. In another study, a modified H120 (R-H120) virus was constructed and was found to elicit a high level of HI antibodies and a comparable protection rate compared with an intact H120-vaccinated group (Zhou *et al.*, 2013). These new generation vaccines can be administered *in ovo* or to live birds, but whether they will increase or reduce the chances of mutation and viral selection pressure and indeed be more economical than existing traditional vaccines requires further studies.

1.7 Laboratory diagnosis

The diagnosis of IBV infections can be done by detection of the virus itself or the specific antibody response against it. The routinely used diagnostic assays for detection of virus are virus isolation (VI), immunofluorescence assay (IFA), immunoperoxidase assay (IPA) and reverse transcription polymerase chain reaction (RT-PCR). Serologically, IBV can be detected by the demonstration of IBV-specific antibodies using various serological tests viz. HI test, the agar gel precipitation test (AGPT), VNT, and the enzyme linked immunosorbent assay (ELISA) (De Wit, 2000).

1.7.1 Detection of virus

1.7.1.1 Virus isolation

IBV can be isolated from infected birds using ECE and chicken tracheal organ cultures (TOCs), preferably from SPF sources (Jackwood & de Wit, 2013). Depending on the history of the disease, samples from the trachea, lungs, kidneys, oviduct and OP, tracheal or cloacal swabs can be required for the isolation of virus (Jackwood & de Wit, 2013). Embryonated SPF eggs and TOCs are reported to be more sensitive than cell cultures, especially from samples involving field strains (Cook *et al.*, 1976b). IBV grows well in the 10-days old SPF ECE, showing higher titres in allantoic fluid harvested between 48 and 60 hours after inoculation (Jackwood & de Wit, 2013). Though direct isolation of virus in conventional primary cell cultures has been proved unsuccessful (Cook, et al., 1976b), adaptation of IBV strains is necessary for replication and induction of observable cytopathic effects (Gillette, 1973; Otsuki *et al.*, 1979). TOCs have also been found to be very successful for isolation of virus from field samples (Cook, 1984). After 3 to 4 days of inoculation, ciliostasis is usually observed, which may vary depending on strains and inoculation dose (Cook, et al., 1976b). However these observations are not sufficient to confirm the presence of IBV; it has to be confirmed by virus antigen detection methods.

1.7.1.2 Detection of virus by antibody-based methods

The AGPT is not expensive, fast and can be easily performed in the laboratory (Lohr, 1981). Though this test requires few laboratory facilities, it is relatively insensitive (De Wit, 2000). In addition, to prevent false negative results caused by

imbalance of the antigen:antiserum ratio, several antisera or an antiserum at different dilutions are required (Lohr, 1981).

The IFA is relatively a cheap and fast technique for detection of IBV antigen in post mortem materials (scrappings of tracheal mucosa or other tissue), cells sedimented from allantoic fluid, TOCs and cell culture. However, it is not always easy to interpret the results, especially with field samples because of non-specific reactions (Jackwood & de Wit, 2013). The IFA can be used as a group-specific test when using IBV-specific polyclonal sera or type-specific by using type-specific monoclonal antibodies (Mabs) (De Wit *et al.*, 1995).

1.7.1.3 Detection of IBV genome

The RT-PCR reaction can been used to detect the presence of IBV genome though; it does not distinguish between infectious and non-infectious virus particles. Therefore, sequences of the RT-PCR product should compared with the corresponding sequences of the vaccine strains (Jackwood & de Wit, 2013). The RT-PCR exhibits a moderate sensitivity when performed directly on samples from infected chickens (Jackwood, et al., 1997), but its sensitivity can be improved by performing one or two passages in embryonated eggs or TOCs to increase viral load followed by detection of the IBV genome by RT-PCR (De Wit, 2000; Jackwood & de Wit, 2013). The primers for RT-PCR are designed near hypervariable regions in the S1 gene and are designed to work with many types of IBV (Adzhar *et al.*, 1996; Worthington, et al., 2008). The primer pairs for group-specific RT-PCR are usually annealed to segments of the IBV genome in the very well conserved regions of viral M and N protein genes (Andreasen *et al.*, 1991; Falcone *et al.*, 1997).

In addition to conventional RT-PCR, another method for detecting IBV genome is through the use of nested RT-PCR. It has been shown to be more sensitive than conventional RT-PCR because of the addition of a second amplification step (Cavanagh, et al., 1999). Owing to high risk of contamination during performing the test and high sensitivity, possibility of getting false positive results as a result of contaminations is high in nested RT-PCR (De Wit, 2000; Jackwood & de Wit, 2013). After detecting the IBV genome, several methods including sequencing the PCR product, subjecting the DNA to restriction enzyme fragment length polymorphism (RFLP) or hybridization using IBV-specific probes have been used to group IBV strains based on genetic characterization (Binns *et al.*, 1985; De Wit, 2000; Jackwood, et al., 1992; Moore *et al.*, 1998).

1.7.2 Demonstration of IBV-specific antibodies

1.7.2.1 VNT

The VNT is the gold standard serotype-specific tests for detection of IBV antibodies, induced primarily by the S1 spike protein. When performing the VNT, appearance of cross-reactions has been reported, especially after multiple contacts with different IBV serotypes (Gelb & Killian, 1987; Karaca & Naqi, 1993). This test can be performed in various test systems *viz.* ECE (Davelaar *et al.*, 1984; Gelb, et al., 1989; Gelb, Wolff*, et al.*, 1991), CEK cells (Hopkins, 1974) and TOCs (Cook, 1984; Darbyshire *et al.*, 1979).

1.7.2.2 HI test

In general, the HI test has been considered to be strain-specific (de Wit *et al.*, 1997; King & Hopkins, 1983; Monreal *et al.*, 1985), therefore its use is limited to monitor response after vaccination. The serotype specificity of the HI test has been reported

to be much lower following re-infection, especially when a heterologous serotype was used for second or subsequent infection (Gelb & Killian, 1987; King & Hopkins, 1983). For instance, the HI response was lower when strain M41 used as antigen in the test following vaccination with H120 as compared to using H120 antigen (de Wit, et al., 1997). Similar results have also been observed wherein, the birds were vaccinated with Mass and 793B types and HI test was performed using M41, 793B, 624/I, It-02 and QX as antigens (Terregino, Toffan, Beato, et al., 2008).

1.7.2.3 ELISA

The ELISA can be used for detection of IBV antibodies after vaccination as well virus infection. By using group-specific ELISA, IBV antibodies can be first detected within 1 week after vaccination or infection (Hawkes, et al., 1983; Mockett & Darbyshire, 1981). Because of the short period between infection and the detection of the first antibodies by ELISA, paired serum sampling is required, one at the first sign of infection and one a week or more later. Delay in first sampling can prevent detection of seroconversion (Jackwood & de Wit, 2013). For IBV-specific antibody detection several ELISA kits are commercially available. The antibody titres detected by ELISA were considerably higher than those obtained by HI (de Wit, et al., 1997) or VN (Marquardt *et al.*, 1981). In general, IBV antibodies can be first detected by ELISA followed by HI test, and last by VNT (Marquardt, et al., 1981; Mockett & Darbyshire, 1981).

1.8 Objectives of the study

New IBV variants constantly emerge across the world that may differ in antigenic properties, tissue tropism and pathogenicity. Little is known about underlying molecular mechanism behind marked difference in pathogenicity by different IBV

genotypes. Though the differences in the tissue tropism and thus differences in the pathogenicity of IBV strains hypothesised to be associated with differences in the binding properties of their spike (S) proteins. Objectives of the work described in the thesis were to 1) study of cellular response to infection by different strains (885, M41, QX and 793B) of IBV in CEK cells and 2) TOCs (Chapter 3, 4 and 6). Further, 3) to study differential immunopathogenesis in chickens (*in vivo*) infected with these IBVs (Chapter 5 and 6). In addition, 4) study on mucosal, cellular and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and the protection conferred against infectious bronchitis virus Q1 strain was undertaken (Chapter 7).

Chapter 2: General materials and methods

In this chapter the general materials and methods used in various experiments have been defined. The procedures only used in specific experiments are described in those relevant chapters.

2.1 Embryonated chicken eggs, cell and organ cultures

i) SPF eggs

The ECE obtained from a commercial source[1] were used for virus propagation, virus titration, *in vitro* CEK cell and TOC preparation as well as a source of day-old SPF chicks for *in vivo* studies. They were incubated at 37.8°C and 55% humidity. The parental flock was free from all major infectious disease agents, including IBV.

ii) Primary CEK cells

Monolayers of primary CEK cells were prepared from kidneys of SPF ECE after 18 day incubation. Kidneys removed aseptically were collected in Minimum Essential Medium (MEM)[2] and 1% antibiotics (penicillin and streptomycin) solution (Appendix I). The kidneys were minced gently in to small pieces with scissors taking care to avoid piercing the gut, followed by trypsinization using 0.25% trypsin solution (Appendix I) for 30 min at 37°C and filtered through a 100 µm mesh to remove tissue debris. After centrifugation at 1500 g for 10 min, cell pellet was resuspended in the growth medium supplemented with 0.5% fetal bovine serum (Appendix I), seeded at $1x10^5$ cells/cm^2 in cell culture plates[3] and incubated at 37^oC with 5% CO_2.

[1] Lohman, Cuxhaven, Germany

[2] Gibco, UK

[3] CELLSTAR, Grenier bio-one, UK

iii) Tracheal organ culture (TOC)

TOC from chicken were prepared using 18-day-old SPF ECE. Briefly, embryos were decapitated; the tracheae were removed under sterile conditions and immediately placed in prewarmed TOC culture medium (Appendix I). The extraneous tracheal tissue was removed using a small scalpel blade and forceps. The trachea was then placed on sterile filter paper[4] and each trachea was cutted into approximately 0.6-mm-thick rings using a tissue chopper[5]. Several rings from the top and the bottom ends were discarded and only one ring was placed per tube[6] with 0.6 ml of prewarmed TOC culture medium. TOCs were cultured at 37°C in a rotating incubator[7] at lowest rotation speed (8 rpm). After 24 hours, the ciliary activity of the respiratory epithelium of each TOC was assessed using an inverted microscope under a low power (100 x magnifications). Only rings with 100% ciliary activity were used for the experiments.

2.2 Viruses and vaccines

All the virus stocks were stored at -70°C and vaccines were kept in refrigerator until used. The different types of field and vaccine strains were used in various experiments; their details are given in the respective chapters.

2.3 Virological methods

i) Propagation of viruses in ECE

The titration of different IBVs was performed using 9 to 11-day-old SPF ECE via the allantoic cavity. At candling eggs were marked around the edge of the air-cell

[4] Fisher scientific, UK

[5] McILWAIN tissue chopper

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

[7] Lab Thermal Equipment, Greenfield, NR OLDHAM

avoiding the area with blood vessels. The marked area was disinfected with 70% alcohol and drilled to make a small hole just above the mark. A 1-ml syringe with a 25-gauge needle was used to inoculate the eggs via the allantoic cavity. The holes were sealed with molten paraffin wax and eggs were incubated at 37^{O}C in an incubator for 3 days. Eggs were examined daily and any embryos dying within 24 hours were discarded. The end points were decided on the basis of IBV specific lesions (death, curling and dwarfing) of the embryos (Figure 2.1).

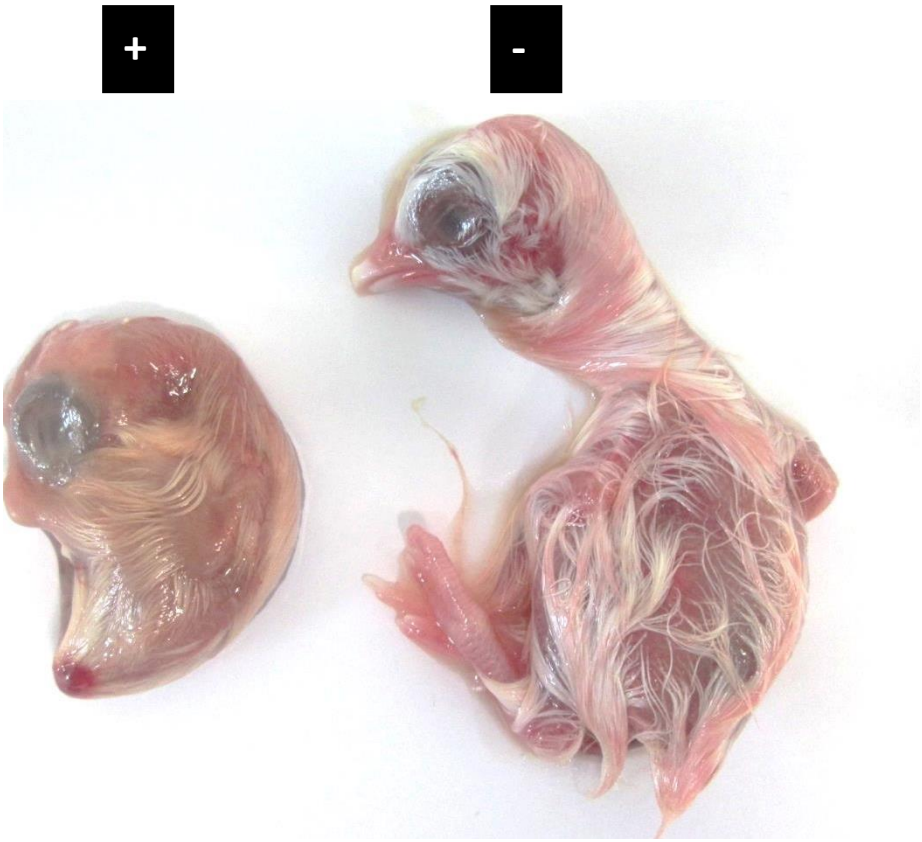


Figure 2.1. Typical IBV lesions (curling and dwarfing) induced after inoculation of ECE with M41 via allantoic cavity. ***Plus*** indicates embryo infected with IBV, ***minus*** indicates non-infected embryo

ii) Propagation of viruses in TOCs

TOCs from 18-day-old ECE were prepared as per method described in 2.1 (iii). The rings with 100% ciliary activity were used for virus propagation. The medium was removed from the tubes and each ring was inoculated with 0.1 ml of the stock virus. The TOCs were incubated at 37^{o}C for one hour to allow virus attachment. After incubation, 0.6 ml TOC culture medium (Appendix I) was added and tubes were further incubated at 37^{o}C in the roller drum. After 5 days, the supernatants were collected, pooled, aliquoted and stored at -70^{o}C until used.

iii) Titration of viruses in ECE and TOCs

To know the definite level of virus in the propagated virus stock, the titration of different IBVs was performed in ECE or TOCs. In brief, three ECE or TOCs rings were inoculated with a tenfold dilution of the virus stock. The eggs were candled after 24 hours of incubation. The embryo mortality occurring up till 24 hpi was considered nonspecific and therefore these eggs were discarded. The ECE were examined for IBV specific lesions (curling and dwarfing) of the embryos up to five days post inoculation. For TOCs, following incubation for up to 7 days at 37^{o}C, complete ciliostasis was taken as the endpoint. The titres were calculated according to the method described earlier (Reed & Muench, 1938) and expressed as the egg infective dose (EID_{50}/ml) or median ciliostasis dose (log_{10} CD_{50}/ml), respectively.

iv) Titration of viruses in CEK cells

To calculate the multiplicity of infection (MOI), all IBV strains were amplified with same passage level and titrated on CEK cells using an immune cytochemical focus assay as described previously (Nelli *et al.*, 2012). In brief, serial 2-fold dilutions of a known volume of TOC supernatants were used to infect CEK cells (prepared as

described above 2.1.ii.) in 96-well cell culture plates[8]. Cells were washed after 2 hours incubation with virus, followed by further 4 hours incubation at 37°C. Cells were then fixed in ice-cold acetone: methanol (1:1) for 10 min. For immune staining, cells were pre-incubated with Tris-buffer saline **(**TBS, Appendix I) for 5 min and endogenous peroxidase activity blocked with 0.03% hydrogen peroxidase[9]. Thereafter, cells were washed 3 times with TBS followed by addition of 50 µl of primary antibody[10] (mouse monoclonal antibody to IBV nucleoprotein diluted 1:500 in TBS). After incubation of the cells at room temperature for 40 min followed by washing with TBS, 50 µl of labelled polymer-HRP[11] was added. The plates were incubated at room temperature for 40 min. Finally, cells expressing the viral nucleoprotein were visualized by using DAB (diaminobenzidine) solution[12] according to the manufacturer's instructions. The cells expressing viral nucleoprotein were counted and the mean number of positive cells in five fields used to calculate focus-forming units of virus per microlitre of inoculum.

2.4 Molecular biology methods

i) Extraction of total RNA

The total RNA from CEK cell pellets, TOC rings and tissue samples was extracted using the RNeasy Mini kit[13]. Briefly, samples were mixed with 600 µl of RLT buffer with mercaptoethanol. This mixture was homogenized using a TissueLyser[14] and a

[8] Grenier bio-one, UK

[9] Sigma-Aldrich, UK

[10] Prionics, Lelystad, The Netherlands

[11] Envision+system-HRP (DAB; Dako, Ely, UK)

[12] Envision+system-HRP (DAB; Dako, Ely, UK)

[13] QIAGEN, UK

[14] QIAGEN, UK

stainless steel beads[15] (5 mm), and lysate was centrifuged at 13000 rpm for 3 min. To the supernatant, 600 µl of 70% ethanol was added, and the solution was transferred to a spin column in two steps. Washing and elution was performed according to the manufacturer's instructions (Appendix I for detailed protocol). The elution volume was 30 µl. Extracted RNA was quantified using NanoDrop[16] 1000 spectrophotometer and stored at -70^{o}C until used for quantitative reverse transcription PCR (qRT-PCR) analysis.

ii) Extraction of viral RNA

Total viral RNA was extracted using the QIAamp viral RNA mini kit[17] as per manufacturer's instructions. Briefly, 140 µl of each sample was mixed with 560 µl of lysis buffer, vortexed briefly and incubated for 10 min at room temperature. Thereafter, 560 µl of 100% ethanol was added to each sample. After mixing gently, the mixture was transferred to spin-cloumn followed by two steps washings with washing buffers (AW-1 and AW-2) (Appendix I). Finally, the RNA was eluted in 60 µl RNase-free water and stored at -70^{o}C till further used.

iii) cDNA synthesis

For further use in host gene expression analysis, first strand cDNA from the total RNA was synthesized using the SuperScript III First-strand synthesis system[18]. A total of 1 µg of the RNA sample was added to reaction mixture I containing random primers, dNTP mix and diethyl-pyrocarbonate (DEPC) treated water (Appendix I).

[15] QIAGEN, UK

[16] Thermo Scientific, Wilmington, DE

[17] QIAGEN, UK

[18] Life Technologies, UK

The mixture was incubated in a thermocycler[19] at 65°C for 5 min, and then placed on ice for at least 1 min. Next 10 µl of cDNA synthesis mix (Appendix I) was added to each RNA/primer mix. The tubes were then centrifuged for 10 s at 5000 rpm, incubated for 10 min at 25°C, followed by 50 min at 50°C and the reaction was terminated at 85°C for 5 min. Finally the tubes were chilled on ice.

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

QRT-PCR of cDNA for the relative expression analysis of selected genes in triplicate was performed on the LightCycler® 480[20], using LightCycler 480 SYBR Green I Master mix[21] and primers listed in Table 2.1. All primers were provided by Eurofins Genomics[22]. A master mix was prepared for each target gene comprising 10 µl of master mix, 1.8 µl each of forward and reverse primers (each at 300nM), and 1.4 µl of nuclease free water. Five micro litres of cDNA diluted at 1:50 was used per reaction in a total reaction volume of 20 µl. The cycling conditions were 10 min at 95°C, 45 cycles of 10 s at 95°C, 10 s at 60°C annealing temperature and 10 s at 72°C, followed by a final step of melting curve analysis to ensure the specificity of the SYBR green PCR. QRT-PCR data was normalised using a relative standard curve method to 18S ribosomal RNA (18SrRNA) expression (Kuchipudi, Tellabati*, et al.*, 2012) and the data were presented as fold difference in gene expression of virus vs mock infected samples or uninfected controls.

[19] GeneAmp PCR system 9700

[20] Roche, UK

[21] Roche, UK

[22] Edersberg, Germany

Table 2.1. Primer abbreviations, full names and sequences

S No.	Primer abbreviation and full name	Primer sequences: sense (S) and anti-sense (AS)
1.Reference gene	18S rRNA (ribosomal RNA)	(S) TGTGCCGCTAGAGGTGAAATT (AS) TGGCAAATGCTTTCGCTTT
2. Viral recognition	LGP2 (Laboratory of genetics and physiology 2)	(S) AGCCCACGAAGCAGTACGA (AS) CGGCAACTCGGGCATCT
	MDA5 (Melanoma differentiation-associated protein 5)	(S) AGGAGGACGACCACGATCTCT (AS) CCACCTGTCTGGTCTGCATGT
	TLR1 (Toll like receptor 1)	(S) GGGAAACCGCTCTGCAGTT (AS) CATTCTTCACCCACAGGGAATC
	TLR2 (Toll like receptor 2)	(S) CCACCGGTCCCTCCTAGTGT (AS) ACCCAACGACCACCAGGAT
	TLR3 (Toll like receptor 3)	(S) GCAATTTCTCCTTCACCTTTTCA (AS) CCTTTATGTTTGCTATGTTGTTATTGCT
	TLR7 (Toll like receptor 7)	(S) GAATTCAAGAGGTTCAGGAACATGA (AS) TTAGGGCAGGGAGTACAAGGATAT
3. Interferons	IFNα (Interferon alpha (Type I))	(S) CTTCCTCCAAGACAACGATTACAG (AS) AGGAACCAGGCACGAGCTT
	IFNβ (Interferon beta (Type I))	(S) TCCAACACCTCTTCAACATGCT (AS) TGGCGTGTGCGGTCAAT
4. Inflammation	IL1β (Interleukin 1 beta)	(S) TGCTGGTTTCCATCTCGTATGT (AS) CCCAGAGCGGCTATTCCA
	IL-6 (Interleukin 6)	(S) CACGATCCGGCAGATGGT (AS) TGGGCGGCCGAGTCT
	NLRP3 (NLR family, pyrin domain containing 3)	(S) TGTGTGTCATCCCTGTCATGAG (AS) TGGTCTTAGAGCACGCAAGGA
5. Other cytokines	IL-8 (Interleukin 8)	(S) CCCTCGCCACAGAACCAA (AS) CAGCCTTGCCCATCATCTTT

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

Quantification of the viral RNA was done by quantitative real-time RT-PCR (RT-qPCR) using the following IBV 3'untranslated region (UTR) gene-specific primer and probe sequences as described previously (Jones *et al.*, 2011): IBVRT1 forward primer CTA TCG CCA GGG AAA TGT C, IBVRT2 reverse primer GCG TCC TAG TGC TGT ACC C, IBVRT3 TaqMan® probe FAM-CCT GGA AAC GAA CGG TAG ACC CT-TAMRA. The qRT-PCR was performed according to the manufacturer's instructions using the One-Step RT-PCR kit[23] and 40 ng of total RNA per reaction (Appendix I). The cycling conditions were 10 min at 50°C and 5 min at 95°C, 40 cycles of 5 s at 95°C, 10 s at 60°C annealing temperature and followed by a final step of 10 s at 72°C. Amplification plots were recorded, analyzed, and the threshold cycle (Ct) determined with the software of Rotor-Gene® Q thermocycler[24]. The Ct values were converted to log relative equivalent units (REU) of viral RNA by a standard curve generated using five 10-fold dilutions of extracted RNA from infective allantoic fluid of 10^6 EID_{50} of M41 as described earlier (Londt *et al.*, 2013).

[23] Qiagen, UK

[24] Qiagen, UK

vi) RT-PCR protocol

The RT-PCR amplification method used in this study was carried out as described previously for IBV (Worthington, et al., 2008) using oligonucleotides as given in Table 2.2.

Table 2.2. Oligonucleotides used in RT- PCR amplification

Step	Oligonucleotides	Sequence (5' to 3')	Gene	Product size (bp)
Nested PCR 1	SX1+	CACCTAGAGGTTTG T/C T A/T GCAT	S1	393
	SX2-	TCCACCTCTATAAACACC C/T TT		
Nested PCR 2	SX3+	TAATACTGG C/T AATTTTTCAGA		
	SX4-	AATACAGATTGCTTACAACCACC		

a) Reverse transcription (RT) reaction

A total of 5 µl of RT reaction mixture containing superscript II RT and one of the negative oligonucleotides (Appendix I) was taken into a 0.5 ml pre-labelled clip top Eppendorf tube[25]. This reaction mixture was overlaid with 2 drops of mineral oil[26] followed by addition of 0.5 µl of RNA under the oil. The RT reaction was carried out in a thermocycler [27] with the following cycle profile: 42^{o}C for 1 hour, 72^{o}C for 10 min and then held indefinitely at 8^{o}C. Positive and negative controls were included in each run. The cDNA was further amplified by using a nested PCR process.

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

[26] Sigma-Aldrich, UK

[27] GeneAmp PCR system 9700

b) Nested PCR 1

The first round PCR (nested PCR 1) was performed in a final volume of 25 µl. The PCR reaction mixture (Appendix I) was thoroughly vortexed and dispensed in 20 µl aliquots under the oil layer in each tube containing the RT-PCR product. The PCR 1 reaction was performed using thermocycler with the following cycle conditions: 94^{o}C for 15 s followed by 35 cycles of 94^{o}C for 10 s, 50^{o}C for 20 s, and 72^{o}C for 40 s and then held indefinitely at 8^{o}C.

c) Nested PCR 2

The total volume of the reaction mixture used for each PCR 2 reaction was 24.5 µl (Appendix I). To 24.5µl of reaction mixture in a 0.5 ml clip top Eppendorf tube, 0.5µl of PCR 1 product was added. After gentle flick and quick spin, the tubes were placed in thermocycler. The nested PCR 2 reaction was performed using following cycle conditions: 94^{o}C for 15 s followed by 35 cycles of 94^{o}C for 10 s, 50^{o}C for 20 s, and 72^{o}C for 40 s and held indefinitely at 8^{o}C.

d) Agarose gel electrophoresis

PCR products were run on a 1.5% w/v agarose gel prepared by dissolving agarose in 1x TBE buffer (Tris-borate-EDTA) (Appendix I). This agarose solution was cooled, stained with 2 µl nucleic acid solution (Redsafe^{TM})[28] and poured into a gel mould (12cm x 9cm) Hybaid Electro-4 gel tank.[29]. Each well in the agarose gel was loaded with 10 µl of product from PCR 2 mixed with 4 µl of loading buffer. A 100 bp ladder (Molecular marker)[30] was also included for comparing the amplicon. The PCR

[28] Intron Biotechnology, Inc

[29] Hybaid Ltd, Middlesex, UK

[30] Amersham Pharmacia Biotech, UK

products were run at 75V for 55 min in 1x Tris-borate-EDTA (TBE) buffer (Appendix I). The gels were viewed and analysed using UV transillumination.

vii) DNA sequencing

The positive nested PCR amplicons (393 bp) were purified with 0.15 µl Exonuclease 1 (EXO) [31] and 0.99 µl shrimp alkaline phosphatase (SAP) [23] at 37°C for 30 min and then at 80°C for 10 min. For direct sequencing, the purified products along with forward primer (SX3+; see table 2.2) were submitted to a commercial laboratory[32]. Alignment of sequences was done by Clustal W (Thompson *et al.*, 1994) and were compared with IBV references available in GenBank (National Centre of Biotechnology Information; http://www.ncbi.nlm.nih.gov/) and BLAST searches were performed to confirm the identification.

2.5 Animals

i) SPF and broiler chicks

The SPF chicks were hatched in our facility (Figure 2.2) from fertile SPF eggs without any previous exposure to IBV i.e. lacking maternally-derived antibodies. The day-old commercial broiler chicks with IBV maternally-derived antibodies were procured from a commercial hatchery[33]. The details of these chicks are included in relevant chapters in which they were used.

[31] 78250, Affymetrix

[32] Source bioscience sequencing, Nottingham, UK

[33] Frodsham Hatchery, Frodsham, UK

Figure 2.2. (A to C) SPF eggs incubated at 37°C for 21 days for hatching. (D) Chicks in an isolation unit on deep litter system

ii) Environment, housing and management for chickens

The chickens were housed in the high containment experimental poultry facility at Leahurst campus (University of Liverpool), with ample access to food and water that was nutritionally complete and appropriate for the age of the chicks. Heat was supplied from an electrical heater which also served as a red light source when the

main lighting was turned off to prevent any cannibalism amongst the birds. The chicks were reared on deep litter in the isolation pens. Appropriate room temperature and humidity was maintained and recorded daily. Approval was given by the University of Liverpool ethical review committee under the project license PPL 40/3723. According to the UK legislation all the correct animal care protocols were in place prior to the experiments. Strict biosecurity measures were in place throughout the experimental period to avoid the risk of cross contamination between different groups. These included footbaths with 2% Virkon[34] disinfectant placed both outside and inside each experimental room and use of protective clothing (overalls, masks, latex gloves and boots). All used gowns, gloves, face masks and carcases were properly disposed of.

2.6 Clinical signs and histopathology

i) Assessment of clinical signs

The experimental chickens were observed daily (for 3 min) for the development of clinical signs. The scoring of clinical signs was done as described previously (Grgic *et al.*, 2008). The clinical signs such as coughing, head shaking and depression of short duration were considered as mild signs, whereas gasping, coughing and depression, along with ruffled feathers were recorded as severe signs.

ii) Histopathology

For histopathological examination, the upper part of the trachea and anterior kidney tissues of IBV infected and control chicks were fixed in 10% buffered formalin. The tissues were embedded in paraffin; sections of 5 µm thickness were cut for haematoxylin and eosin (H&E) staining. The histopathological changes in

[34] Antec International, Suffolk, UK

trachea and kidney were examined and scored based on visible changes as follows: 0 = no change, 1= mild, 2 = moderate, 3 = severe (Chen & Itakura, 1996)

iii) Examination of gross lesions

Gross lesions were examined on all dead or scarified chicks and were scored according to the scoring system as described in Table 2.3.

Table 2.3. Scoring system of gross lesions

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

2.7 Collection of samples

i) Blood

Blood samples were collected from the wing vein of each chick using a 23-gauge sterile needle[35] and 2 ml syringe[8]. For serology, after collection blood was kept individually in labelled bijou tubes without anticoagulant. To allow separation of serum, the tubes were placed in slanting position overnight at room temperature. Next day, sera was separated by centrifugation at 3,000 g for 10 min and stored at -20^{o}C until used. To determine the percentage of T-lymphocyte subpopulations, blood was collected in tubes with heparin[36] (at final concentration of 10 USP/ml of blood).

ii) Tissues

Following euthanasia of bird using of 0.5-1 ml of Euthatal[37] through wing vein injection, tissue samples were collected aseptically and placed in labelled bijou tubes. The samples were securely transported to the laboratory and processed as soon as possible. In addition, severely affected challenged and all the remaining chickens at the end of each experiment were humanely killed by using the same method of euthanasia.

[35] Becton, Dickinson and Co. Ltd

[36] Sigma-Aldrich, UK

[37] Pentobarbitone sodium B.P., Rhone Merieux, Ireland

2.8 Analysis of cell death rate in CEK cells

i) MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reduction) assay

The MTT assay was performed using CellTiter® 96, a non-radioactive cell proliferation assay kit[38] as per manufacturer's instructions. CEK cells were seeded on cell culture plates at a density of $1x10^5$ cells/cm^2. After incubation at 37°C for 48 hours, 80-90% confluent monolayers were washed three times with PBS, and then mock-infected with the growth medium supplemented with 0.5% fetal bovine serum (Appendix I) or infected with different IBV strains at MOI of 1.0 in total volume of 100 µl. Plates were incubated at 37°C for 24 hours. After incubation, 15 µl of the dye solution was added to each well and plates were further incubated at 37°C for 4 hours. Finally for extracting the formazan produced in the cells, 100 µl of solubilisation solution/stop mix was added to each well. Absorbance values were measured using multi plate reader[39] at 570 nm with gentle shaking before reading to ensure uniformly coloured solution.

ii) Apoptosis by flow cytometry

The CEK cells were seeded on cell culture plates at a density of $1x10^5$ cells/cm^2. After incubation at 37°C for 48 hours, 80-90% confluent monolayers were washed three times with PBS, and then mock-infected with the growth medium supplemented with 0.5% fetal bovine serum (Appendix I) or infected with different IBV strains at MOI of 1.0 in total volume of 100 µl. Cells were harvested at 24 and 48 hpi. Briefly, cells were washed in PBS and removed from the cell culture plate by

[38] Promega, Madison, WI, USA

[39] Thermo Scientific, Multiskan FC, Finland

treatment with 0.25% trypsin solution (Appendix I), followed by washing of cells using growth medium containing serum (Appendix I) to neutralize the trypsin. The cells were then washed, resuspended in binding buffer[40], and subsequently analyzed by staining with ApoAlert® Annexin V kit[41] according to the manufacturer's instructions using flow cytometer[42]. Fluorescence was quantified using CFlow software. The single-dye positive controls were prepared by staining representative cells with Fluorescein isothiocyanate (FITC) and propidium iodide (PI) dye individually and used for estimation of proper colour compensation. The unstained cell sample was used as a negative control to adjust the threshold.

2.9 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay for detection of apoptotic cells in TOCs

Detection of apoptotic cells in paraffin sections of TOC was performed with TUNEL assay using the peroxidase (POD) *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN, USA) according to the manufacturer's instructions. A total of five sections were analyzed for each time point. After de-waxing, the TOC sections were incubated with 0.1 % Triton X-100[43] in 0.1% sodium citrate[44] at room temperature for 8 min. After washing 3 times with PBS, sections were overlaid with 50 µl TUNEL reaction mixture and incubated in a humidified atmosphere for 60 min at 37°C in the dark. After washing the specimen 3 times with PBS, 50 µl Converter-POD was added and slides were incubated in a humidified atmosphere

[40] Clontech, UK

[41] Clontech, UK

[42] BD Accuri C6, San Jose, CA

[43] Sigma-Aldrich, UK

[44] Sigma-Aldrich, UK

for 60 min at 37°C. Following incubation, sections were washed thrice with PBS and peroxidase reaction was developed using the DAB Peroxidase Substrate kit[45] according to the manufacturer's instructions. Sections were counter stained with hematoxylin[46] for 1 min and mounted with Aquatex[47].

2.10 Serology

i) ELISA

To detect specific anti-IBV antibodies serum samples were tested with a commercial IBV ELISA IDEXX kit[48], according to the manufacturer's instructions. In brief, a dilution of 1:500 of serum samples was made in sample diluent buffer. One hundred µl of the diluted samples were pipetted into the antigen coated plates and incubated at room temperature for 30 min. Thereafter, plates were washed 3-5 times with de-ionized water followed by addition of 100 µl of goat anti-chicken conjugate to each well. After incubation of the plates at room temperature for 30 min followed by washing with de-ionized water, 100 µl of the tetramethylbenzidine (TMB) substrate solution was added to each well. The plates were incubated for 15 min in the dark. Finally, the reaction was stopped by adding 100 µl of stopping solution to each well. Absorbance values were measured using multi plate reader[49] at 650 nm. For the results to be valid the mean of the negative control should be less than 0.150 and the difference between the positive control mean and negative control mean should be 0.075. The samples with sample to positive (S/P) ratio

[45] Vector Laboratories, UK

[46] Merck, Germany

[47] Merck, Germany

[48] Ofddrop, The Netherlands

[49] Thermo Scientific, Multiskan FC, Finland

greater than 0.2 were considered positive. S/P ratio was used for calculation of titres using formula Log_{10} titre = 1.09 (log S/P) + 3.36.

ii) Haemagglutination inhibition (HI)

The Q1 HA antigen for HI test was prepared in our laboratory as described earlier (King & Hopkins, 1983). The Q1 HA antigen was prepared from allantoic virus which was propagated in ECEs as described above in 2.3.1. The virus was concentrated from the allantoic fluid by ultracentrifugation[50] at 30,000 g (Rotor SW 41 Ti) for 90 min at 4°C. The virus pellet was resuspended to 1/100 of the original volume in 2-hydroxy-ethyl-piperazine-N2-ethane sulphonic acid (HEPES) buffer (Appendix 1). This concentrated virus was treated with bacterial phospholipase C type I[51] (1 unit/ml) for 2 hours at 37°C and used as HA antigens in a HI test.

The HI test was conducted according to standard procedures (OIE), using 4 HA units of antigen per well. Prior to the HI test, back-titration of the HA antigen was carried out to ascertain the 4 HAU. HI tests were carried out in microtiter "U" shaped plates[52]. Two fold serial dilutions (starting from 1:2) of 25 µl of serum samples was prepared in PBS followed by addition of 25 µl 4HA antigen. The plate was incubated at room temperature for 30 min. After that, 25 µl of 1% RBC suspension[53] was added to each well. After mixing by gentle tapping plate was kept at room temperature for 40-60 min. HA was easily determined by tilting the plate to observe the presence of tear shaped streaming of the RBC's in control wells. The HI

[50] Beckman Coulter, USA

[51] Sigma-Aldrich, UK

[52] Thermo Fisher Scientific, Denmark

[53] Sci-Tech Laboratories, UK

titres were read as the reciprocal of the highest dilution showing complete inhibition and the HI geometric mean titres were expressed as reciprocal $\log_2$.

2.11 Statistical analysis

All the parametric data were analysed using the one-way analysis of variance (ANOVA), followed by the *post-hoc* least significant difference (LSD) multiple comparison test. Differences between groups were considered significant at $P<0.05$. For only the trachea and kidney lesion score and viral load the significant differences between the groups were analysed by Kruskal-Walis test followed by Dunn's mean test. Differences between groups were considered significant at $P<0.05$. The Kruskal-Wallis test is considered an acceptable alternative to ANOVA, as it also looks to compare between different groups at different time point, but for nonparametric data instead. All the statistical analysis was done using GraphPad Prism version 6 software.

Chapter 3: Comparative study of *in vitro* cellular response to infection by different strains of infectious bronchitis virus

The data from this Chapter and Chapter 4 has been submitted (Appendix III) in peer-reviewed journal as below:

- Rajesh Chhabra, Suresh V Kuchipudi, Julian Chantrey, and Kannan Ganapathy. (2015) Pathogenicity and tissue tropism of infectious bronchitis virus correlates to elevated apoptosis and innate immune responses. Virology **488** (1): 232-241.

3.1 Introduction

Infectious bronchitis (IB) is an acute and highly contagious disease affecting chicken of all ages and is characterized by lesions in respiratory and urogenital organs (Cavanagh, 2007; Dolz *et al.*, 2006) caused by a gamma coronavirus. Avian infectious bronchitis virus (IBV) continues to cause serious economic losses in chicken production globally. Along with highly pathogenic avian influenza virus (HPAIV) and velogenic Newcastle disease virus (NDV) infections, IB is the most economically relevant viral respiratory disease of poultry industry (Cook, et al., 2012). Vaccination has been considered as the most reliable approach for controlling IBV infection (Meeusen *et al.*, 2007). However, current vaccines are proven to be inadequate in controlling IBV due to constant emergence of new variant viruses (De Wit, 2000; de Wit, et al., 2011). Concurrent circulation of both classic and variant IBVs has been identified in most parts of the world raising major challenges to global prevention and control efforts.

In past two decades the majority of the serotypes/genotypes that have emerged appear to be endemic (Bochkov, et al., 2006). IBV QX was first reported in China in 1996 from birds with proventriculitis (YuDong, 1998). Later QX was reported from Russia and then to Europe and rapidly spread to become the most widespread recent genotype of non-vaccine origin (Bochkov, et al., 2006; Worthington, et al., 2008). In the UK, IBV QX-like was isolated from proventriculus of broilers with poor FCR and body weight gain also showing respiratory signs (Ganapathy, et al., 2012). This genotype of IBV mainly affects the respiratory and reproductive systems (Ducatez, et al., 2009; Terregino, Toffan, Beato, et al., 2008). In addition to these lesions, the QX-like serotype has been associated with proventriculitis (YuDong,

1998) severe kidney damage (Ganapathy, et al., 2012; Liu & Kong, 2004; Terregino, Toffan, Beato, et al., 2008; Worthington, et al., 2008) and false-layer syndrome (de Wit, et al., 2011; Landman, 2005).

In the winter of 2000, a severe outbreak of renal disease occurred in several broiler farms, causing great economic loss to the Israeli broiler industry as a result of mortality and poor weight gain of the birds. The causative agent was determined to be a nephropathogenic infectious bronchitis virus (NIBV) by virus isolation from kidneys and molecular characterization of the isolate, designated IS/885/00 and protection studies have shown that Mass vaccines provide inadequate protection against this novel variants (Meir *et al.*, 2004). Later on in Egypt in addition to circulation of Massachusetts, 793B, QX and Dutch strains, the strains similar to 885 was reported by (Abdel-Moneim *et al.*, 2006). Of late, 885 has also been detected in France and Ukraine (Ganapathy, personal communication), and Libya (Awad *et al.*, 2014).

Despite the economic importance of IBV and decades of research into the pathogenesis of infection, significant gaps in our knowledge exist. The field cases reported until now suggest that these strains have tropism for many organs and high pathogenicity, which provides a unique opportunity to dissect the molecular mechanisms contributing these differences. Differences in the tissue tropism and thus differences in the pathogenicity of IBV strains have been hypothesised to be associated with differences in the binding properties of their spike proteins (Casais, et al., 2003; Wickramasinghe *et al.*, 2011). While the ability to bind to susceptible host cells is the first step in virus life cycle, host innate immune responses could also be important in the pathological outcome of IBV infection. Variant IBVs

constantly emerge and the host determinants of IBV pathogenicity are not yet fully understood. Early host innate immune responses and apoptosis of infected cells *in vitro* could be useful indicators for predicting the pathological outcome of viral infection *in vivo*. For example the three different genotypes of NDV produce distinct host response patterns in chicken spleenocytes which is useful to differentiate the NDV genotypes (Hu *et al.*, 2012). However, the pattern of virus infection in *vitro* or *ex vivo* to differentiate IBV strains with different tissue tropism is not fully understood. In order to establish a host response signature to predict the tissue tropism and pathogenicity of IBVs, we investigated apoptosis and innate immune responses in chicken embryo kidney (CEK) cells following infection with 885, QX and M41 IBV strains.

3.2 Materials and methods

3.2.1 Cell Culture

Monolayers of primary CEK cells were prepared from kidneys of SPF ECE as described in Chapter 2.1.ii.

3.2.2 Viruses

To widen the range of IBV strains, in the present study, we used a traditional strain, M41, and two variant IBV strains, IS/885/00-like and QX-like (Table 3.1). IS/885/00 causes mortality, poor weight gain and severe renal damages (Meir, et al., 2004), while QX causes economic problems in most of Europe and other parts of the world through renal and reproductive problems (de Wit et al., 2012). In this study, IS/885/00-like isolate (885) (Awad *et al.*, 2013) and a UK QX isolate, strain KG3P (Ganapathy, et al., 2012) were used. These viruses were isolated in SPF ECE as described in in Chapter 2.3.i from either tracheal or kidney tissue collected from

field cases. All IBV strains were propagated in chicken TOCs prepared from 19 to 20-dayold SPF ECE as per method described in Chapter 2.3.ii. (Cook, et al., 1976b). For CEK cell infection, all IBV strains were titrated on CEK cells using an immune cytochemical focus assay as described in Chapter 2.3.iv (Nelli, et al., 2012).

Table 3.1 Details of infectious bronchitis viruses used in this study

Virus	Source	Passage level
Israeli variant IS/885	Received as third passage AF from the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy	AF went through four further passages in ECEs and 2 passages in TOCs
QX	Isolated in our laboratory from proventriculus from a flock of 56-do commercial broiler chickens	Went through five passages in ECEs and 2 passages in TOCs
Massachusetts type M41	From Prof. R C Jones, Leahurst	Obtained after numerous number of passages in ECEs and 2 passages in TOCs.

3.2.3 Virus infection of CEK cells

CEK cells were seeded on cell culture plates at a density of $1x10^5$ cells/cm^2. After incubation at 37°C for 48 hours, 80-90% confluent monolayers were washed three times with PBS, and then infected with different IBV strains. Initially, to assess the effect of viral infection on primary CEK cells, cultured CEK cells were infected with different IBV strains at a range of MOI from 0.25 to 4.0 and quantified the cell metabolic activity at 24 and 48 hpi using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide reduction (MTT) assay. Based on the preliminary results, finally CEK cells were infected with different IBV strains at a MOI of 1.0 or mock-infected with cell culture medium (Appendix I). Triplicate wells were used for each virus or mock infection for each time point. Cells were analyzed at 24 and 48

hpi to determine the effect of virus infection on cell metabolic activity, induction of apoptosis, and cell culture supernatant were titrated for infectious virus. At 9 and 24 hpi cell pellets were collected in RLT buffer and total RNA was extracted using RNeasy Mini kit as described in Chapter 2.4.i followed by cDNA synthesis as described in Chapter 2.4.iii. The cDNA were used for the quantification of host gene expression analysis.

3.2.4 Measurement of cell metabolic activity by MTT assay

CEK cells in 96-well cell culture plates were analysed for metabolic activity at 24 and 48 hpi using CellTiter 96, a non-radioactive cell proliferation assay according to procedure described in Chapter 2.8.i.

3.2.5 Analysis of apoptosis by flow cytometry

CEK cells at 24 and 48 hours after virus or mock infection were analysed to quantify percent of apoptosis by flow cytometry as described in Chapter 2.8.ii.

3.2.6 Quantification of infectious virus

Supernatants from virus infected or mock-infected CEK cells were titrated for infectious virus in TOCs following method described in Chapter 2.3.iii and expressed as CD_{50}/ml (Cook, et al., 1976b).

3.2.7 Host gene expression analysis

Quantitative reverse transcription PCR (qRT-PCR) of cDNA samples in triplicate was performed on the LightCycler® 480 using LightCycler 480 SYBR Green I Master mix (Roche, UK) as described in Chapter 2.4.iv. QRT-PCR data was normalized using a relative standard curve method to 18S ribosomal RNA (18SrRNA) expression (Kuchipudi, Tellabati, et al., 2012) and the data were presented as fold difference in gene expression of virus vs mock-infected samples.

3.2.8 Statistical analysis

The data were analysed using one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test using GraphPad Prism version 6 software LSD. Differences between groups at that time point were considered significant at $P<0.05$.

3.3 Results

CEK cells infected with IBV strains 885, QX, M41 at MOI 1.0 or mock-infected were subjected to immuno-cytochemical staining of viral nucleoprotein at 6 hpi. The dose used resulted in similar level of infectivity in CEK cells across all the three virus strains (Figure 3.1)

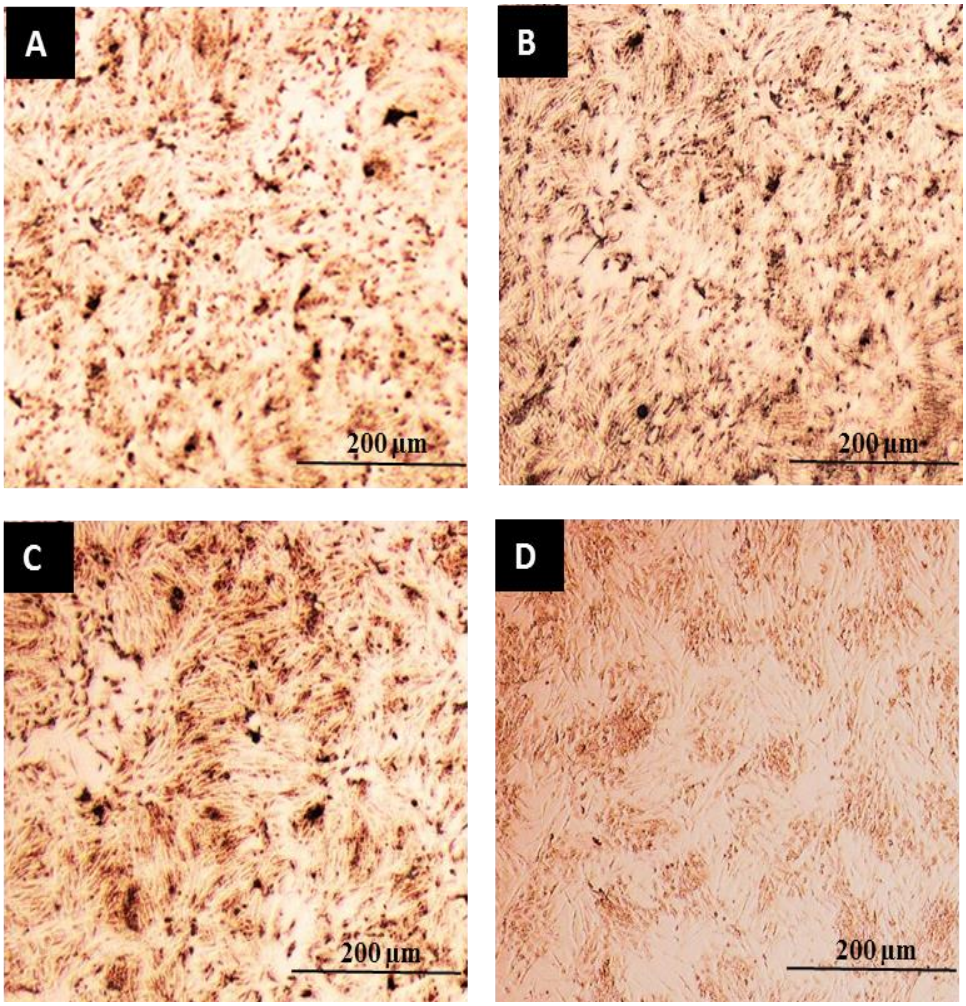


Figure 3.1. CEK cells infected with (A) 885, (B) QX, (C) M41 or (D) mock-infected and immuno-cyto chemical staining of viral nucleoprotein at 6 hpi revealed similar levels of infection of all the three viruses.

3.3.1 MTT assay

In cell metabolic activity assay using MTT, we found a significant (*P<0.05*) reduction in the cell metabolic activity in IBV infected CEK cells compared with mock-infected cells both at 24 and 48 hpi (Figure 3.2). The pattern of reduction in cell metabolic activity was dose dependent both at 24 (Figure 3.2, A) and 48 hpi (Figure 3.2, B) by all the three viruses, since with increasing level of infection greater reductions in cell metabolic activity was noticed. In addition, at 48 hpi, significantly lower (*P<0.05*) metabolic activity was observed in CEK cells infected by all the three strains than at 24 hpi (Table 3.1).

At MOI 1.0, after 24 hpi cell metabolic activity of CEK cells infected by 885 and QX showed no significant (*P<0.05*) difference and were overall significantly lower (*P<0.05*) than M41 infected cells (Figure 3.3). Thereafter, at 48 hpi, cell metabolic activity of CEK cells infected with 885 was overall significantly lower (*P<0.05*) followed by QX and M41 (Figure 3.3).

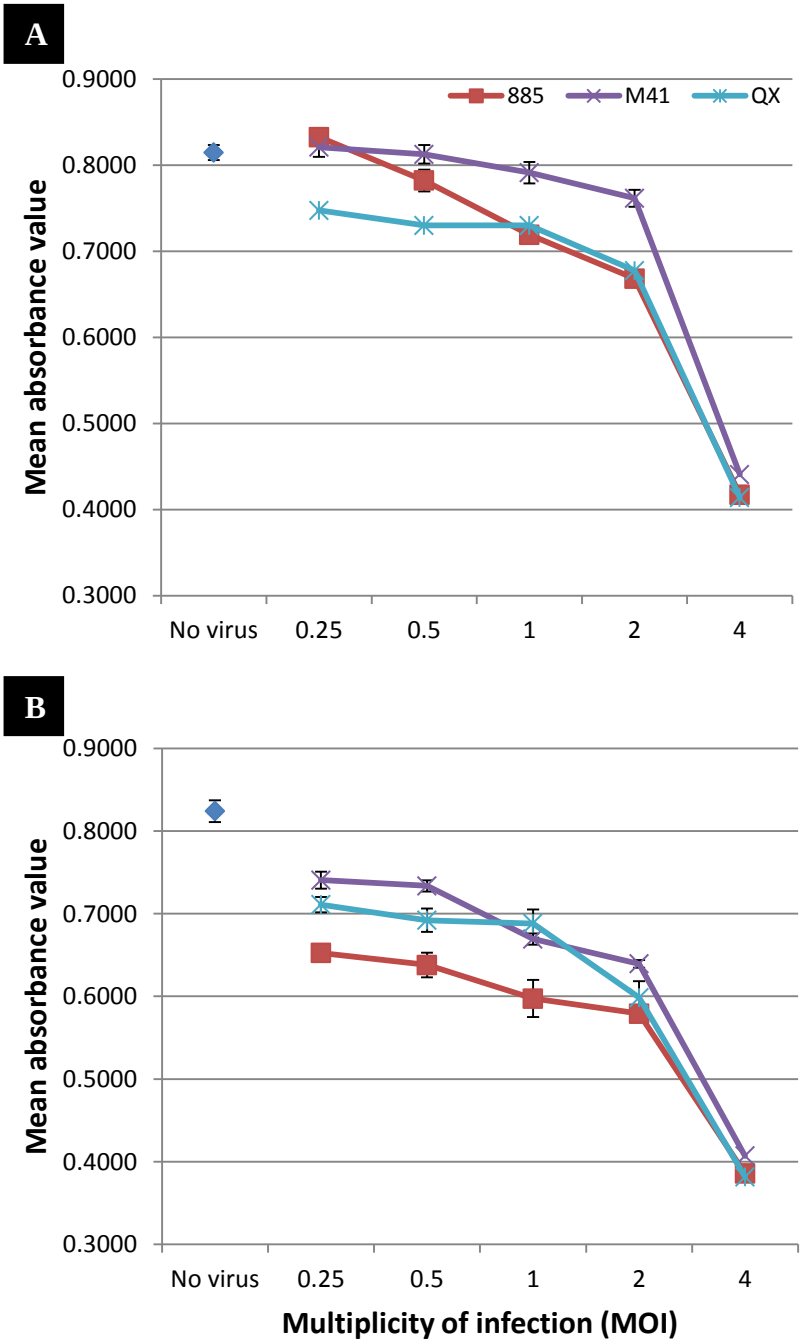


Figure 3.2. Measurement of cell metabolic activity of CEK cells, using an MTT assay, infected with different strains of IBVs, with a range of MOI from 0.25 to 4.0, at (A) 24 hpi (B) 48 hpi. Data represents mean of triplicate wells with error bar showing standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test (*P<0.05)*.

Table 3.1. Mean absorbance value by MTT assay of primary CEK cells infected with different IBV strains 885, QX, M41 or mock-infected

(Values are Mean±SE)

MOI	24 hours post infection				48 hours post infection			
	Mock-infected	**885**	**M41**	**QX**	**Mock-infected**	**885**	**M41**	**QX**
0.25	0.8146 ± 0.012^{B}	0.8326 ± 0.088^{A}	$0.8210\pm0,004^{B}$	0.7475 ± 0.004^{C}	0.8241 ± 0.013^{A}	0.6525 ± 0.022^{E}	0.7407 ± 0.004^{C}	0.7110 ± 0.014^{D}
0.5	0.8146 ± 0.012^{A}	0.7822 ± 0.009^{B}	0.8126 ± 0.008^{A}	0.7300 ± 0.011^{C}	0.8241 ± 0.013^{A}	0.6379 ± 0.008^{D}	0.7336 ± 0.010^{B}	0.6921 ± 0.009^{C}
1.0	0.8146 ± 0.012^{A}	0.7192 ± 0.013^{D}	0.7913 ± 0.008^{B}	0.7301 ± 0.010^{D}	0.8241 ± 0.013^{A}	0.5974 ± 0.015^{D}	0.6694 ± 0.006^{C}	0.6882 ± 0.014^{B}
2.0	0.8146 ± 0.012^{A}	0.6682 ± 0.017^{D}	0.7616 ± 0.020^{B}	0.6776 ± 0.012^{D}	0.8241 ± 0.013^{A}	0.5791 ± 0.022^{C}	0.6393 ± 0.006^{B}	0.5986 ± 0.017^{C}
4.0	0.8146 ± 0.012^{A}	0.4173 ± 0.012^{C}	0.4408 ± 0.019^{B}	0.4140 ± 0.009^{C}	0.8241 ± 0.013^{A}	0.3857 ± 0.010^{C}	0.4070 ± 0.004^{B}	0.3816 ± 0.019^{C}

** Different letter indicate statistically significant difference* (*P<0.05*)

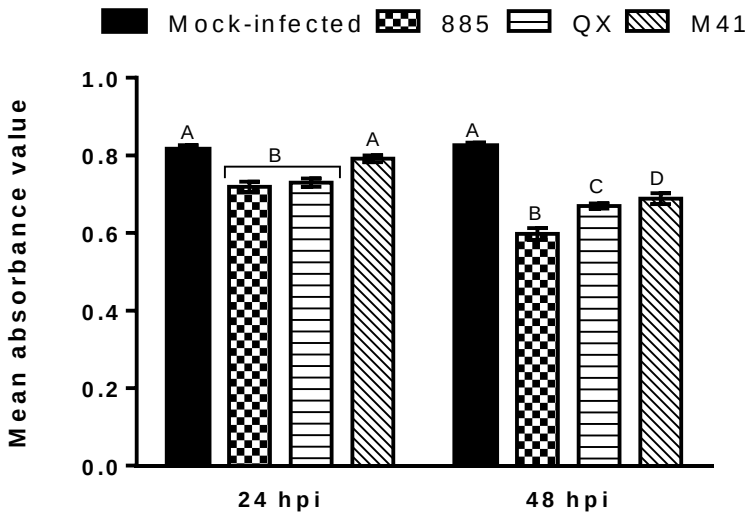


Figure 3.3. IBV induced cell metabolic activity in CEK cells. The cells were infected with 885, QX, M41 at MOI 1.0 or mock-infected and analyzed using MTT asssay at designated time points after infection. Data represents mean of triplicate wells with error bar showing standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

3.3.2 Flow cytometry analysis of apoptosis

In cell metabolic activity assay using MTT, was observed a reduction in the cell metabolic activity in IBV infected CEK cells compared with mock infected cells. Therefore, we examined whether the differences in the cell metabolic activity were due to different levels of apoptosis induction. The CEK cells were infected with different strains of IBV at MOI 1.0 or mock-infected and analysed for apoptosis at 24 and 48 hpi. We found a significantly (*P<0.05*) increased in apoptosis in IBV infected CEK cells compared with mock-infected cells both at 24 and 48h after virus infection (Figure 3.4). Notably, infection of CEK cells with IBV strains 885 and QX resulted in significantly (*P<0.05*) higher apoptosis than M41 both at 24 and 48 hpi (Figure 3.4).

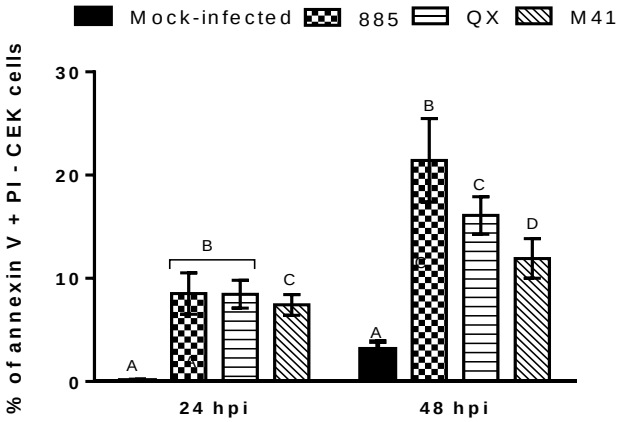


Figure 3.4. IBV induced apoptosis in CEK cells measured by flow cytometry. CEK cells were infected with different strains of IBVs at an MOI of 0.1 or mock-infected and analysed for apoptosis at designated time points after infection. Data represents mean of triplicate wells with error bar showing standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

3.3.3 Viral output in cell culture supernatant

To determine the difference in the level of infectious virus produced following infection with different IBVs, we titrated the cell culture supernatants in the TOCs. Interestingly, IBV strains 885 or QX infection resulted in significantly (*P<0.05*) lower infectious virus production from CEK cells at 24 and 48 hpi compared with M41 infected cells (Figure 3.5).

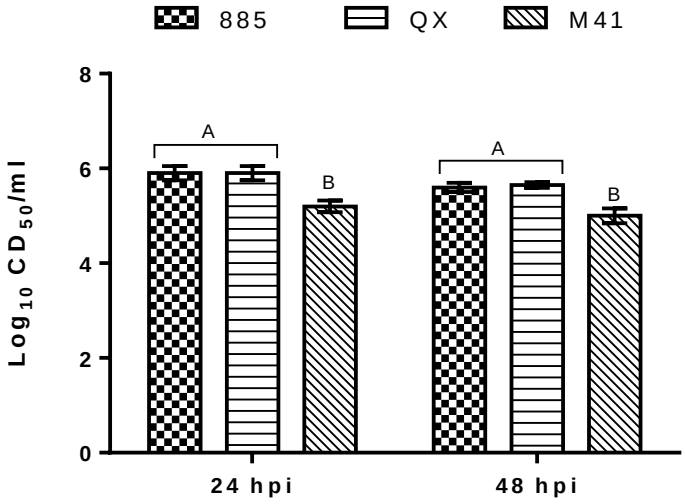


Figure 3.5. CEK cells were infected with the indicated viruses at an MOI of 1.0. Virus production in the cell culture supernatants were determined in chicken embryo TOCs at the indicated times post infection. Data show the mean values (n=3) with standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

3.3. 4 Host gene expression

Next, various host genes involved in the response to the IBV infections were analyzed for alterations in mRNA levels in CEK cells.

3.3.4.1 Transcripts involved in viral recognition

The mRNA expression data for LGP2, MDA5, TLR1, TLR2, TLR3 and TLR7 in CEK cells are illustrated in Figure 3.6. The LGP2 mRNA expression was significantly *(P<0.05)* down-regulated at 24 hpi in M41 and QX infected groups, but there was no significant change in expression level in 885 infected group (Figure 3.6 A). For MDA5 mRNA expression, a significant up-regulation *(P<0.05)* was observed at 9 hpi in all IBV infected groups compared with mock-infected group, with no significant *(P<0.05)* difference between expression level of 885 and QX whereas, it was significantly higher than M41 (Figure 3.6 B). The MDA5 mRNA expression was significantly down-regulated *(P<0.05)* at 24 hpi in all IBV infected groups compared with mock-infected (Figure 3.6 B). At 9 hpi, there was no significant *(P<0.05*) difference in the TLR1 (Figure 3.6, C), TLR2 (Figure 3.6 D) and TLR7 (Figure 3.6 F) mRNA expression levels when comparing IBV infected groups to the mock-infected group. Expression of TLR1 and TLR2 mRNA were also seen to have a significant down-regulation *(P<0.05)* compared with mock-infected group at 24 hpi. However, there was no significant *(P<0.05*) change of expression of mRNA for TLR7 at 24 hpi. Conversely, TLR3 mRNA expression (Figure 3.6 E) was significantly *(P<0.05)* up-regulated in 885 and QX infected groups than M41 at 9 hpi, whereas at 24 hpi, a significant down-regulation *(P<0.05)* was seen in QX infected group.

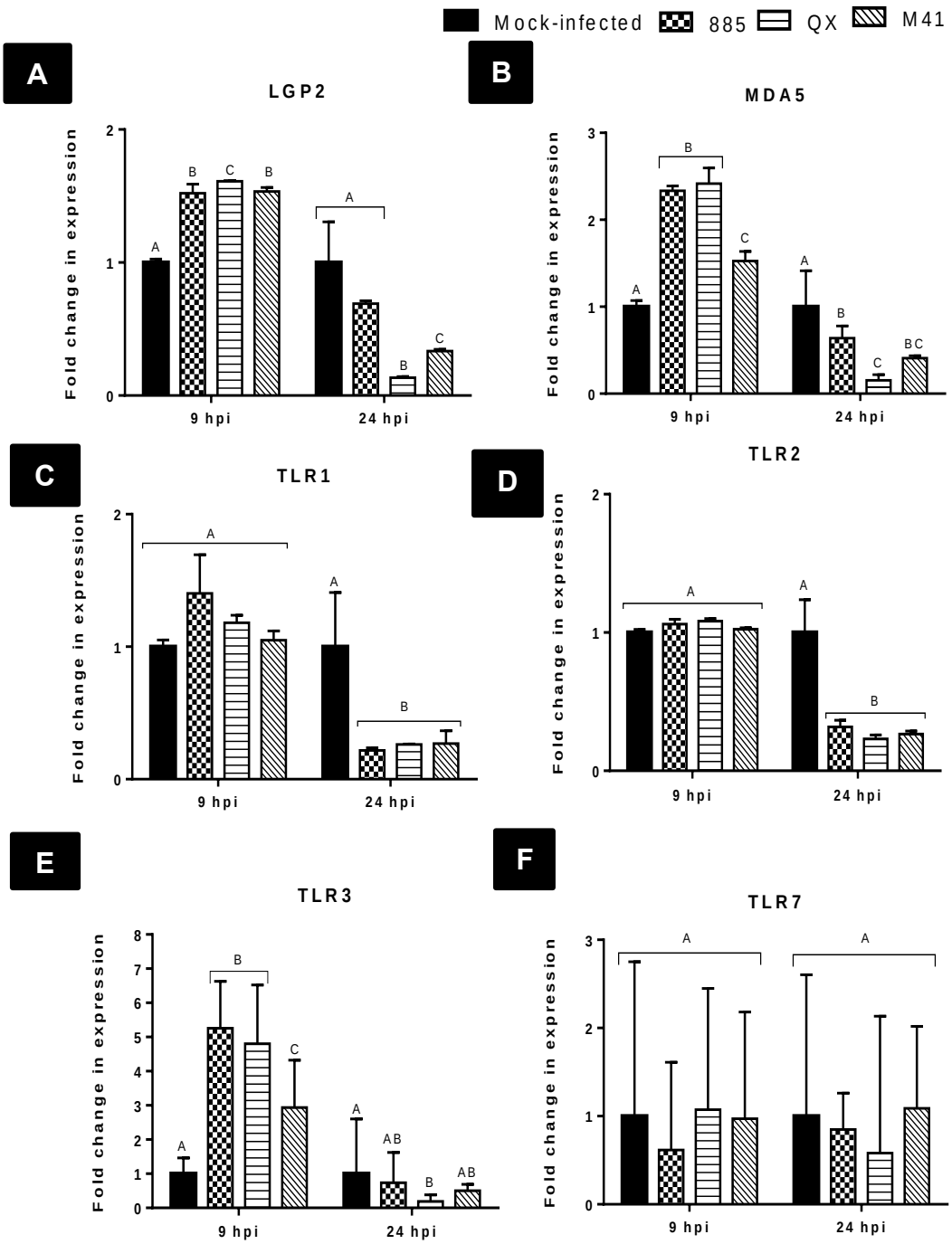


Figure. 3.6. Relative mRNA expression of (A) LGP2, (B) MDA5, (C) TLR1, (D) TLR2, (E) TLR3, (F) TLR7 in CEK cells infected with IBV strains 885, QX, or M41 at MOI 1.0. Relative mRNA expression was determined by real-time PCR normalised to 18S rRNA. Graphed values are the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

3.3.4.2 Interferons, Cytokines and related transcripts

The mRNA expression data for INF-α and INF-β is illustrated in Figure 3.7 A & B respectively. There was no significant *(P<0.05)* change in INF-α mRNA expression levels at 9 hpi in any of the infected groups compared with mock-infected group, whereas at 24 hpi, a significant *(P<0.05)* down-regulation was seen in all infected groups compared with mock-infected group (Figure 3.7 A). At 9 hpi, expression level of INF-β mRNA significantly *(P<0.05)* up-regulated in 885 infected group followed by QX, whereas no significant *(P<0.05)* change was observed in M41 infected group compared with mock-infected group (Figure 3.7 B). At 24 hpi, a significant *(P<0.05)* down-regulation was seen in all infected groups compared with mock-infected group.

Regarding mRNA expression of major pro-inflammatory cytokines involved in innate immune response such as IL-1β (Figure 3.7, D), IL-8 (Figure 3.7 E), and the inflammation associated NLRP3 (Figure 3.7 F) seen to have no significant change at 9 hpi and trend of down-regulation compared with mock-infected group was noticed at 24 hpi *(P<0.05)* (Figure 3.7). IL-6 mRNA expression at 9 hpi was found to have significant *(P<0.05)* up-regulation only in 885 infected group, whereas at 24 hpi, there was no significant change in mRNA expression in any infected group (Figure 3.7 D).

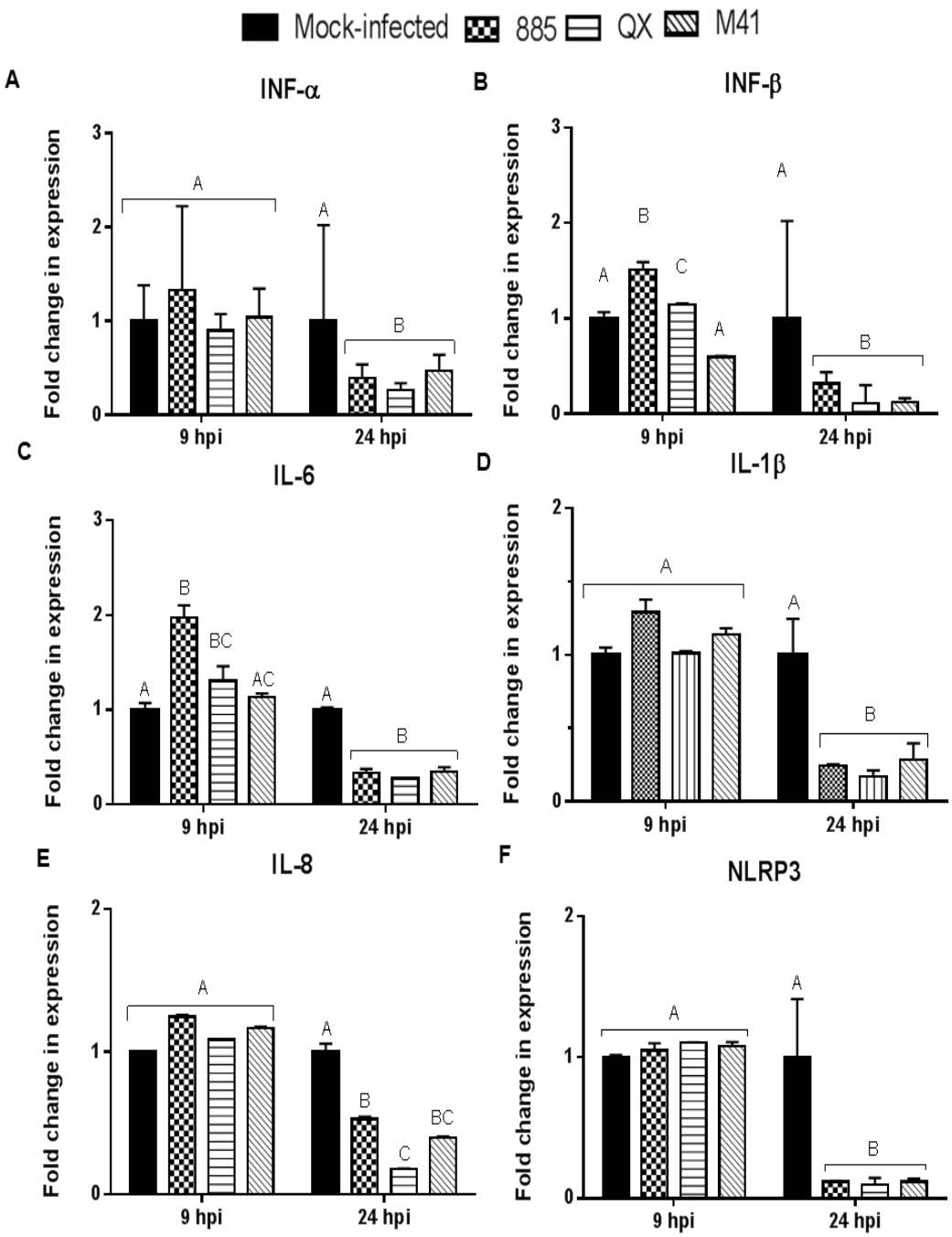


Figure. 3.7. Relative mRNA expression of (A) INF-α, (B) INF-β, (C) IL-6, (D) IL-1β, (E) IL-8, (F) NLRP3 in CEK cells infected with IBV strains 885, QX, or M41 at MOI 1.0. Relative mRNA expression was determined by real-time PCR normalised to 18S rRNA. Graphed values are the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

3.4 Discussion

Though the primary target organ of IBV is the epithelial surface of the respiratory tract, the virus also infects several non-respiratory tissues, such as the gastrointestinal tract, the oviduct, and the kidney (Ganapathy, et al., 2012). Infection and replication in the kidney might cause mild to severe lesions depending on the IBV strain (Delmas, 1990). To investigate the role of apoptosis and innate immune responses, in order to institute a host response signature to envisage the tissue tropism and pathogenicity of IBVs, chicken embryo kidney (CEK) cells were infected with IBV strains 885, QX and M41.

To assess the effect of infection on CEK cells, we infected CEK cells with different IBV strains and further quantified the metabolic activity of cells, using MTT assay. Overall a significantly higher death rate of CEK cells was observed in all infected groups compared with mock-infected and, as was expected, the effect was dose dependent, with increasing levels of infection inducing greater cell death rate. Similar phenomena of dose dependent cell death rate was observed with canine coronavirus (CCoV), wherein, at 48 hpi in A-72 cells proliferation rate was reduced by two/three times compared to the uninfected control cells at two different MOI chosen and was dose dependent (Ruggieri *et al.*, 2007).

A variety of viruses have been shown to induce apoptosis or programmed cell death in host cells (Clarke & Tyler, 2009; Shen & Shenk, 1995; Teodoro & Branton, 1997). IBVs are known to induce apoptosis in infected cells and B-cell lymphoma 2 (Bcl-2) family proteins modulate IBV-induced apoptosis (Li, et al., 2007; Liu, et al., 2001; Zhong, Liao, et al., 2012; Zhong, Tan, et al., 2012). The modulation of Bcl-2 family proteins during IBV infection has also been postulated to be under the

regulation of signalling pathways such as endoplasmic reticulum (ER) stress and Mitogen-Activated Protein Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) pathways (Fung & Liu, 2014; Zhong, Liao, et al., 2012). Further, IBV infection results in a growth-inhibitory effect by inducing cell cycle arrest at S and G_2/M phases (Li, et al., 2007) both of which results in apoptosis. Therefore, due to the difference in induction of cell death following infection with different IBV strains in CEK cells, we investigated the potential role of apoptosis as a cause of this phenomenon at 24 and 48 hpi.

Our findings indicate that more rapid cell death, with features of apoptosis was higher in CEK cells infected with 885 and QX than M41. Apoptosis is a cell-death programme that is initiated in response to various extrinsic and intrinsic signals such as cellular stress, including virus infection, and plays an important role in the pathogenesis of many viruses (Wang *et al.*, 2007). Should this phenomenon occur *in vivo* in case of IBV, apoptosis and cellular destruction of kidney tissue are correlated. Earlier work by (Benyeda *et al.*, 2010), reported that mild histopathological lesions in kidney were recorded in infection of chickens with M41 and 793B strain, while the nephropathogenic effect of the QX-like strains was greater and confirmed the high affinity of this strains for the kidney. The potential for apoptosis to cause such tissue damage has also been recognized for the Coronavirus infections, wherein, apoptosis in different cell types was found to be consistent with the clinical observation of apoptosis in different organs infected by SARS-CoV (Tan *et al.*, 2004).

The IBV strains 885 and QX resulted in significantly higher up-regulation of innate immune sensing genes TLR3 and MDA5 along with higher IFN-β mRNA levels in CEK

cells at 9 hpi than M41 infection. Concurrent with our findings, a previous study observed a significant increase of TLR3 mRNA expression in both trachea and lungs of IBV infected chickens when compared to the uninfected controls (Kameka, et al., 2014). We did not find any particular pattern of regulation of genes namely TLR1 and TLR2.

TLR3 and TLR7 are well known for recognition of RNA virus encoded pathogen associated molecular patterns (PAMPs) (Akira, 2001). TLR7 was not significantly affected by IBV infection in CEK cells. The end products of TLR3 signalling pathway is the production of anti-viral type I interferon (IFN)-α and -β, whereas and TLR7 activation results in the production of pro-inflammatory cytokines (Guillot *et al.*, 2005). Concurrent with TLR3 activation, we found a significant up-regulation of IFN-β but not IFN-*α* mRNA expression at 9 hours after IBV infection in CEK cells. Likewise, lack of TLR7 induction correlated with no change in the expression of pro-inflammatory cytokines namely IL-1β, IL-8 and NLRP3 in IBV infected CEK cells. In contrast to our findings, a recent study found that IFN-β transcription was not found in CEK cells until 12 hours after infection with IBV M41 at MOI of 0.1 (Kint, et al., 2015). This difference may have been due to differences in the MOI used and origin of cells. Notably, expression of all the innate immune genes tested, was down-regulated at 24 hours after IBV infection in CEK cells. This could be a consequence of host shut-off induced by IBV virus replication. Coronaviruses are known to down-regulate host gene expression and host mRNA degradation in order to suppress host innate immune response (Kamitani *et al.*, 2006; Tohya *et al.*, 2009). Corona virus nucelocapsid (N) protein has been shown to interfere with the 2',5'-oligoadenylate synthetase/RNaseL (2'–5' OAS) activation, which is responsible

for Type I IFN induction and can also inhibit the production of various pro-inflammatory cytokines and chemokines via global translational shutdown (Ye *et al.*, 2007; Zhong, Tan, et al., 2012).

Several *in vitro* and *ex vivo* studies have suggested a key role of TLR3 in viral detection (Alexopoulou *et al.*, 2001; Bowie & Haga, 2005; Finberg & Kurt-Jones, 2004; Tabeta *et al.*, 2004). However, recent evidence suggests TLR3 contributes to detrimental effects of viral infections. TLR3 mediates West Nile virus entry into the brain, causing lethal encephalitis (Wang *et al.*, 2004) and contributes to a detrimental inflammatory response to influenza virus infection in mice resulting in acute pneumonia (Le Goffic, et al., 2007). A recent study showed that MDA5, but not TLR3, is involved in the sensing of IBV (Kint, et al., 2015). However, higher mRNA levels of MDA5 and TLR3 correlated with the *in vivo* pathogenicity of the IBV strains used in this study, such that more nephropathogenic strains like 885 and QX induced much higher levels in CEK cells. It is possible that MDA5 activation is a protective antiviral host response against the virus whereas TLR3 contributes the detrimental effects of the viral infection. The precise role of MDA5 and TLR3 in host defence against IBV infection and/or disease pathogenesis is not yet fully understood and hence, warrants further in-depth studies.

The LGP2 expression was significantly increased at 9 hpi but was down-regulated at 24 hpi in CEK cells. LGP2 functions as a negative regulator by interfering with the recognition of viral RNA by RIG-I and MDA5 (Yoneyama *et al.*, 2005). MDA5 is IFN-inducible (Kang *et al.*, 2004); once IFN response is triggered, this innate antiviral loop initiates autoamplification of MDA5 until the natural inhibitor LGP2 is induced (Yoneyama, et al., 2005). However, we found that the expression of LGP2, MDA5

and IFN-β mRNA levels were down-regulated at 24 hpi, possibly due to the virus induced host gene shutoff. Similar to our findings, a recent study also reported a down-regulation of TLR3, IL-1β and IFN-γ expression in IBV infected chicken trachea at 12 hpi (Kameka, et al., 2014). Cells were infected at MOI of 1.0 in this study; infection at a lower MOI could be helpful to profile the effect of LGP2 on MDA5 and IFN-β regulation in IBV infected cells.

Interestingly, IBV strains 885 or QX infection resulted in significantly lower infectious virus production from CEK cells at 24 and 48 hpi compared with M41 infected cells. In fact, in case of virus-infected cells, the induction of cell death by apoptosis can either reduce viral spread in the host by early killing of infected cells, or facilitate viral progeny dissemination, through apoptotic bodies, which hides the virus antigen and limits induction of inflammatory and immune response (Koyama *et al.*, 2000). Notably, in our study higher apoptosis and IFN-β expression correlated with significantly lower infectious virus production from CEK cells. This could be due to increased antiviral state induced by IFN-β and/or increased apoptosis. Apoptosis of cultured cells have previously been shown to be associated with reduced production of infectious influenza virus in duck cells (Kuchipudi, Dunham*, et al.*, 2012).

In summary, IBV strains 885 and QX induced stronger innate immune (TLR3, MDA5 and IFN-β) and cell death responses (apoptosis) than M41 in CEK cells. This *in vitro* evidence indicates that higher apoptosis together with elevated levels of TLR3, MDA5 and IFN-β expression correlates to pathogenicity of IBVs in kidney (CEK cells) tissues. This study also highlights that this host response signature could be useful

to make a predictive prognosis of the tissue tropism of novel IBV strains using CEK cells.

Chapter 4: Difference in innate immune response and apoptosis in chicken tracheal organ culture infected with IBV strains of varying virulence

The data from this Chapter and Chapter 3 have been submitted (Appendix III) in peer-reviewed journal as below:

- Rajesh Chhabra, Suresh V Kuchipudi, Julian Chantrey, and Kannan Ganapathy. (2015) Pathogenicity and tissue tropism of infectious bronchitis virus correlates to elevated apoptosis and innate immune responses. Virology **488** (1): 232-241.

4.1 Introduction

Infectious bronchitis is an acute and highly contagious disease pathogen affecting chickens of all ages and causes lesions in respiratory and urogenital organs (Cavanagh, 2007; Dolz, et al., 2006; Jackwood & de Wit, 2013). It causes enormous economic problems in poultry flocks that are often difficult to control by current vaccines or vaccination programmes that are based on the prevalent IBV strains in the field (Sjaak de Wit, et al., 2011). However, the constant emergence of new IBV variants complicates the control of the disease. The IBV isolates present a high antigenic diversity (Hofstad, 1975) and emergent strains that differ in antigenic properties, tissue tropism and pathogenicity are continuously being reported across the world (Gelb, Lunt*, et al.*, 1991; Jackwood, 2012; Shaw *et al.*, 1996; Zanella *et al.*, 2000). While all IBV strains appear to infect the birds via the respiratory tract, they subsequently reach secondary sites for further replication and persistence. An example is IBV strain M41, which though primarily replicates in the respiratory tract, is also able to replicate in a range of tissues. In contrast, other strains of IBV such as strain IS/885/00 are primarily nephropathogenic. IS/885/00, referred as a nephropathogenic infectious bronchitis virus (NIBV), was first isolated from a severe outbreak of renal disease in several broiler farms in Israel (Meir, et al., 2004). In recent years, IBV strain QX, which mainly affects the respiratory and reproductive systems, is widespread among poultry flocks in many countries (Ducatez, et al., 2009; Terregino, Toffan, Beato, et al., 2008). In addition, QX-like serotypes have also been associated with proventriculitis (YuDong, 1998) and severe kidney damage (Ganapathy, et al., 2012; Liu & Kong, 2004; Terregino,

Toffan, Beato, et al., 2008; Worthington, et al., 2008) suggesting their broad tissue tropism.

Cook & Huggins (1986) noted that the evaluation of the pathogenicity of IBV strains for the respiratory tissue has been difficult to quantify. Cubillos *et al.* (1991) suggested differences in virulence of IBV strains for the trachea based on the variability of the tracheal damage in terms of ciliary activity in unvaccinated chickens. Raj and Jones (1996a) also reported little difference among several IBV strain s using measurement of ciliary activity as a criterion for damage to the tracheal epithelium. Later a study on comparative analysis of the sialic acid binding activity of four different IBV strains demonstrated that sialic acid binding is important for the tracheal epithelium by all four strains used in the study (Abd El Rahman, et al., 2009). It was also reported that the strains differed in their efficiency to infect the tracheal epithelium.

Interactions between viruses and hosts occur at two levels: viral capacity to gain access to the target cell and competition between the viruses and host cells to control the cellular protein synthesis machinery. The virus/host interactions are largely determined by the virulence of the pathogen and the host innate immune response (Schneider & Shenk, 1987). In addition, occurrence of cell death by apoptosis has been observed during infection with IBV (Li, et al., 2007; Liu, et al., 2001; Zhong, Liao, et al., 2012; Zhong, Tan, et al., 2012). Since IBV is highly variable therefore, studying IBV alone to develop control strategies against IB outbreaks is not sufficient (Sjaak de Wit, et al., 2011). As viruses and hosts show coevolution, so enhancing our knowledge of host responses against IBV will be helpful to protect the poultry industry from the menace of IB. Based on these findings, we

hypothesize that early host innate immune responses and apoptosis of infected TOCs could be useful indicators for predicting the pathological outcome of viral infection *in vivo*. In this study comparative analysis of changes in viral growth kinetics, induction of apoptosis and the early host innate immune responses at host transcriptional level after infection of chicken TOCs with 885, QX and M41 strains of IBV was undertaken.

4.2 Materials and methods

4.2.1 Tracheal organ culture

TOCs were prepared from tracheas of SPF ECE as described in Chapter 2.1.iii.

4.2.2 Viruses

To cover a range of different virus strains in the present study we have used a pathogenic M41, the currently important IBV variant IS/885/00 (Meir, et al., 2004) and a QX variant (de Wit et al., 2012) Details of the viruses are described in Chapter 3.2.2). All IBV strains were propagated in chicken TOCs prepared from 19 to 20-dayold SPF ECE as per method described in Chapter 2.3.ii. (Cook, et al., 1976b). For TOCs infection, all IBV strains were titrated on TOCs as described in Chapter 2.3.iii and expressed as CD_{50}/ml (Cook, et al., 1976b).

4.2.3 Virus infection of TOCs

TOCs were infected 5 days after preparation to allow the early inflammatory responses of the tissue to subside (Reemers *et al.*, 2009). TOCs were infected with IBV strains using a dose of 4 $\log_{10}$ CD_{50} per individual ring or mock-infected with TOC medium (Appendix I) without virus. Five rings were used for each virus or mock infection for each time point. The infection dose of 4 $\log_{10}$ CD_{50} was chosen based on a preliminary study wherein, this dose caused 100% ciliostatsis in TOC by day 3

of infection. For quantification of ciliostasis, TOC were examined at 24 and 48 hpi under an inverted microscope to determine the relative ciliary activity (RCA). Samples of TOC supernatant or complete rings were collected at 9, 24 and 48 hpi. TOC rings fixed in 4% paraformaldehyde (PFA) (Appendix I) were analysed for apoptotic cells using *in situ* Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay and supernatants were titrated for quantification of infectious virus at 24 and 48hpi. Total RNA was extracted from TOC rings collected at 9 and 24 hpi using RNeasy Mini kit as described in Chapter 2.4.i followed by cDNA synthesis as described in Chapter 2.4.iii. The cDNA were used for the quantification of host gene expression analysis.

4.2.4 Quantification of ciliostatsis

The proportion of the periphery of the tracheal rings with ciliary beating was assessed at every point time and expressed as peripheral ciliary activity (PCA). The ciliary vigour was scored as follows: 3 for fast harmonic beating, 2 for moderate co-ordinated beating, 1 for slow in coordinated beating and 0 for lack of beating as described previously (Gabridge *et al.*, 1974; Stadtlander *et al.*, 1991).The relative ciliary activity (RCA) was calculated by multiplying the PPCA to ciliary vigour. Time taken in dpi for IBV strains to cause a 50 % reduction in RCA was calculated using polynomial regression equation (3^{rd} order). All the TOCs used in this study had a RCA of 100% before infection.

4.2.5 Detection of apoptotic cells in TOCs

Apoptotic cells were detected and quantified by the TUNEL assay using the peroxidase (POD*) in situ* cell death detection kit according to the manufacturer's instructions as described in Chapter 2.9. A total of five sections were analyzed for

each time point. The number of apoptotic cells was counted microscopically in each TOC ring and was presented as mean of the total apoptotic cells per complete ring.

4.2.6 Quantification of infectious virus

Supernatants from virus infected or mock-infected TOCs were titrated for infectious virus in TOCs following method described in Chapter 2.3.iii and expressed as CD_{50}/ml (Cook, et al., 1976b).

4.2.7 Host gene expression analysis

The transcription of selected innate immune genes such as TLR3, TLR7, INF-α and IFN-β was quantified. Quantitative reverse transcription PCR (qRT-PCR) of cDNA samples in triplicate was performed on the LightCycler® 480 using LightCycler 480 SYBR Green I Master mix (Roche, UK) as described in Chapter 2.4.iv. QRT-PCR data was normalized using a relative standard curve method to 18S ribosomal RNA (18S rRNA) expression (Kuchipudi, Tellabati, et al., 2012) and the data were presented as fold difference in gene expression of virus vs mock-infected samples.

4.2.8 Statistical analysis

The data were analysed using one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test using GraphPad Prism version 6 software LSD. Differences between groups at that time point were considered significant at $P<0.05$.

4.3 Results

4.3.1 Quantification of ciliostatsis

The time taken for a 50 % reduction in RCA of TOCs infected with different strains of IBVs is illustrated in Figure 4.1. No difference in the time taken (1.70 dpi) for a 50 % reduction in RCA was found between the viruses.

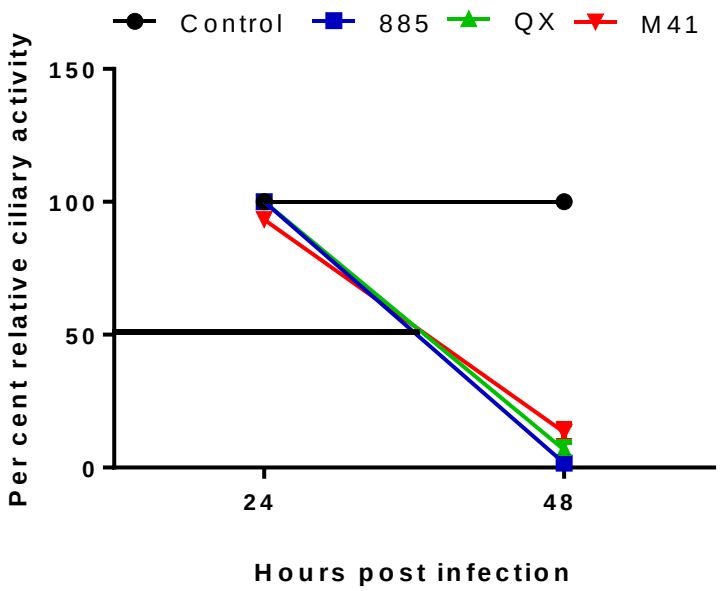


Figure 4.1. Comparison of relative ciliary activity of chicken trachea organ cultures (TOCs). TOCs were infected with the indicated viruses using a dose of 4 $\log_{10}$ CD_{50} per individual ring or mock-infected.

4.3.2 Apoptotic cells in TOCs

Total apoptotic cells in IBV or mock infected TOCs were evaluated by TUNEL assay (Figure 4.2). We found that IBV infection resulted in significant increase in total number of apoptotic cells in TOCs compared with mock-infected controls at 24 and 48 hpi. Notably, infection of TOCs with IBV strains M41 resulted in significantly (*P<0.05*) higher apoptosis compared with those infected with 885 or QX both at 24 and 48 hpi (Figure 4.3).

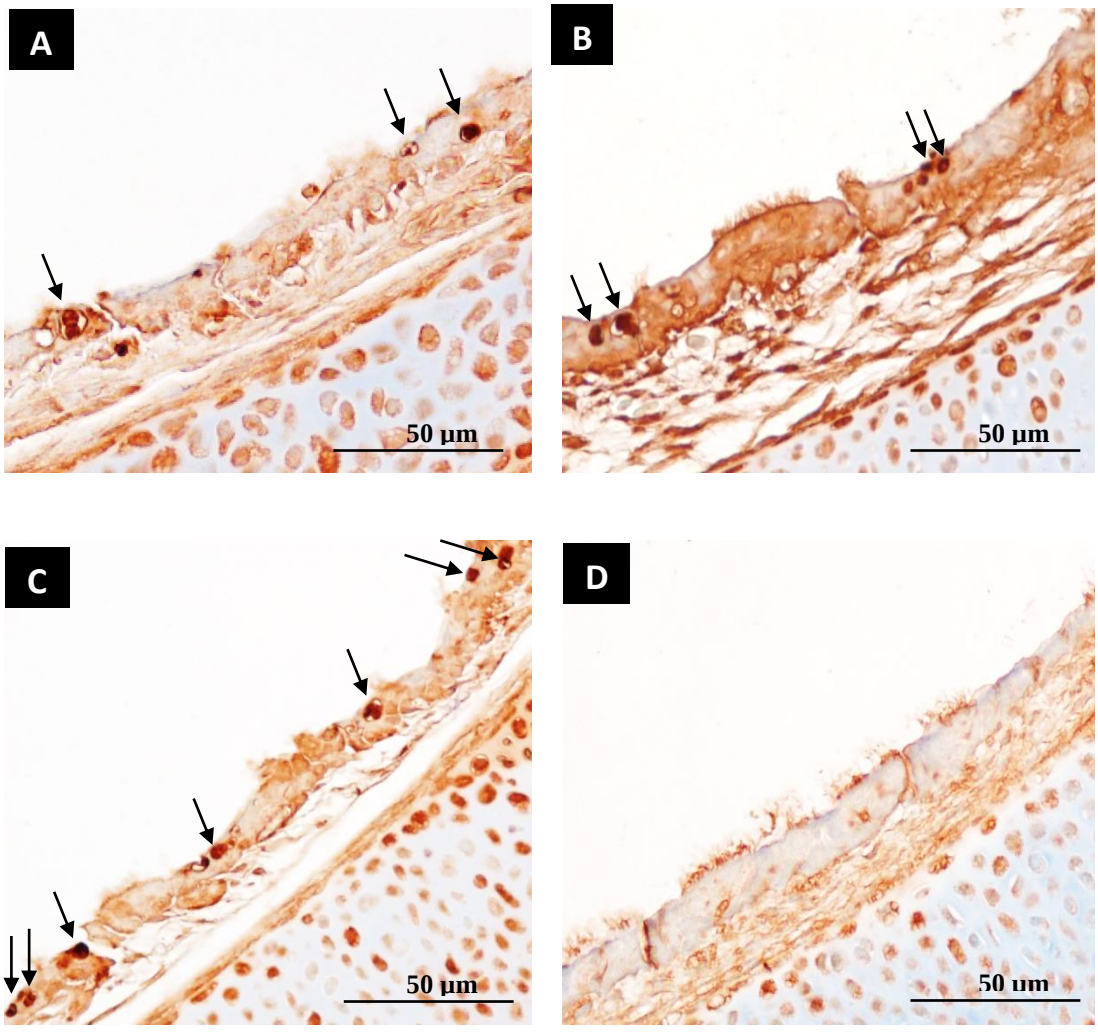


Figure 4.2. *In situ* Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) staining of chicken embryo tracheal organ cultures (TOCs) infected with (A) 885, (B) QX (C) M41 or (D) mock-infected at 24 hours post infection (hpi). Apoptosis TUNEL staining of TOCs infected with IBVs showing multiple apoptotic cells with degeneration and loss of cilia. Arrows indicate apoptotic cells.

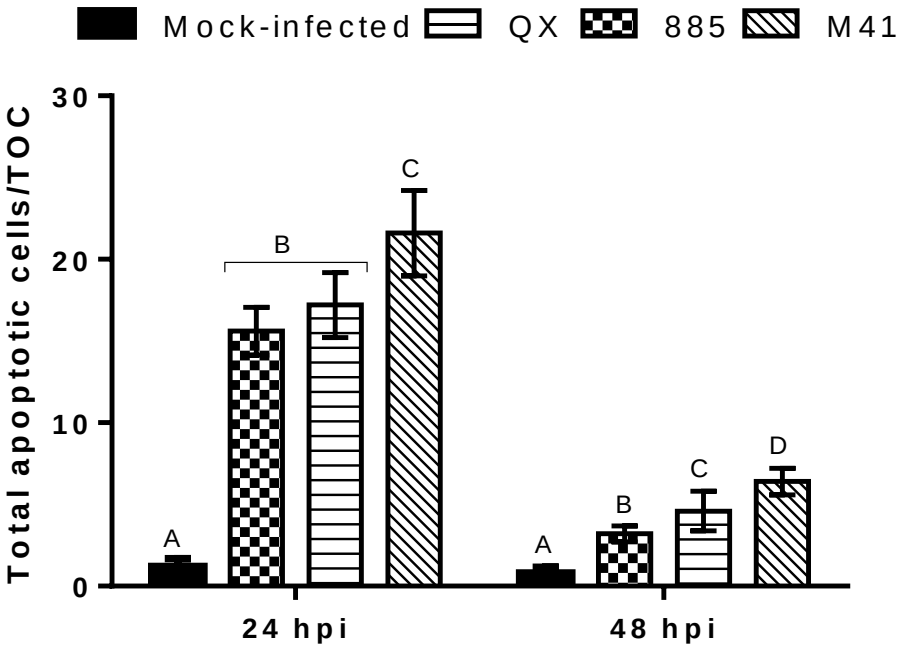


Figure 4.3. Total apoptotic cells in TOCs evaluated by TUNEL assay. TOCs were infected with IBV strains 885, QX, M41 or mock infected and evaluated at 24 and 48 hpi. Data represents mean (n=5) with error bar showing standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

4.3.3 Viral output in TOC supernatant

To determine the difference in the level of infectious virus produced following infection with different IBVs, we titrated the supernatants in the TOCs. Interestingly, IBV strains M41 infection resulted in significantly (*P<0.05*) lower infectious virus production from TOCs at 24 and 48 hpi compared with 885 or QX TOCs (Figure 4.4).

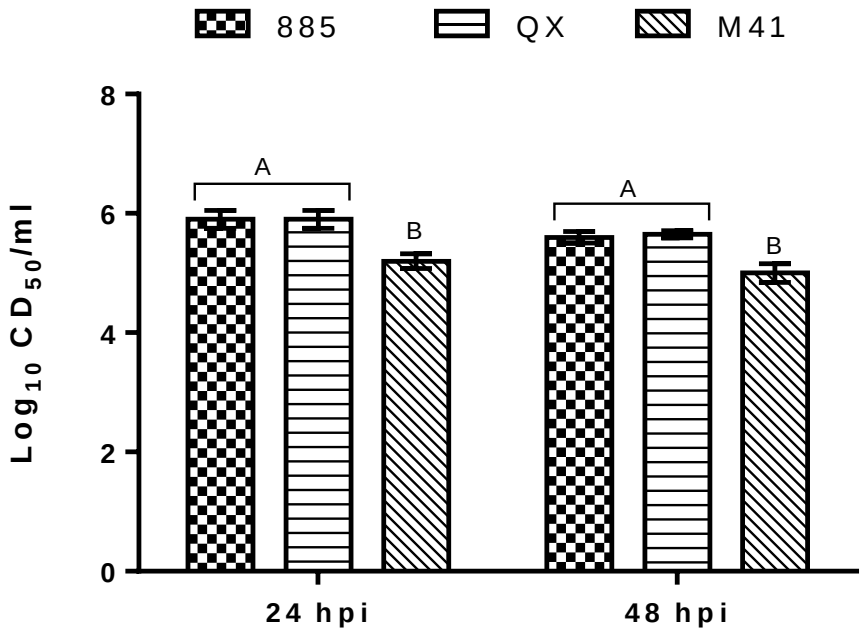


Figure 4.4. TOCs were infected with the indicated viruses using a dose of 4 $\log_{10}$ CD_{50} per individual ring or mock-infected. Virus production in the TOC supernatants were determined in chicken embryo TOCs at the indicated times post infection. Data show the mean values with standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

4.3.4 Host gene expression

The mRNA expression data for INF-α and INF-β are illustrated in Figure 4.5 A & B, respectively. There was no significant *(P<0.05)* change in INF-α mRNA expression levels at 9 hpi and 24 hpi in any of the infected groups compared with the mock-infected group. At 9 hpi, expression level of INF-β mRNA was significantly *(P<0.05)* up-regulated in the M41 infected group compared with 885 or QX. Conversely, the INF-β mRNA expression was significantly *(P<0.05)* higher at 24 hpi in M41 and QX infected groups compared with 885.

TLR3 mRNA expression (Figure 4.5, C) was significantly *(P<0.05)* up-regulated in M41 infected group than 885 and QX at 9 hpi, whereas at 24 hpi, mRNA expression level in all infected groups was significantly (*P<0.05*) up-regulated compared with mock-infected group but without any significant (*P<0.05*) difference between the infected groups.

For MDA5 mRNA expression, a significant up-regulation *(P<0.05)* was observed at 9 hpi in all IBV infected groups compared with mock-infected group, whereas it was significantly higher *(P<0.05)* in M41 infected group than 885 or QX (Figure 4.5, D). Thereafter, at 24 hpi, MDA5 mRNA expression M41 infected group was overall significantly higher (*P<0.05*) followed by QX and 885.

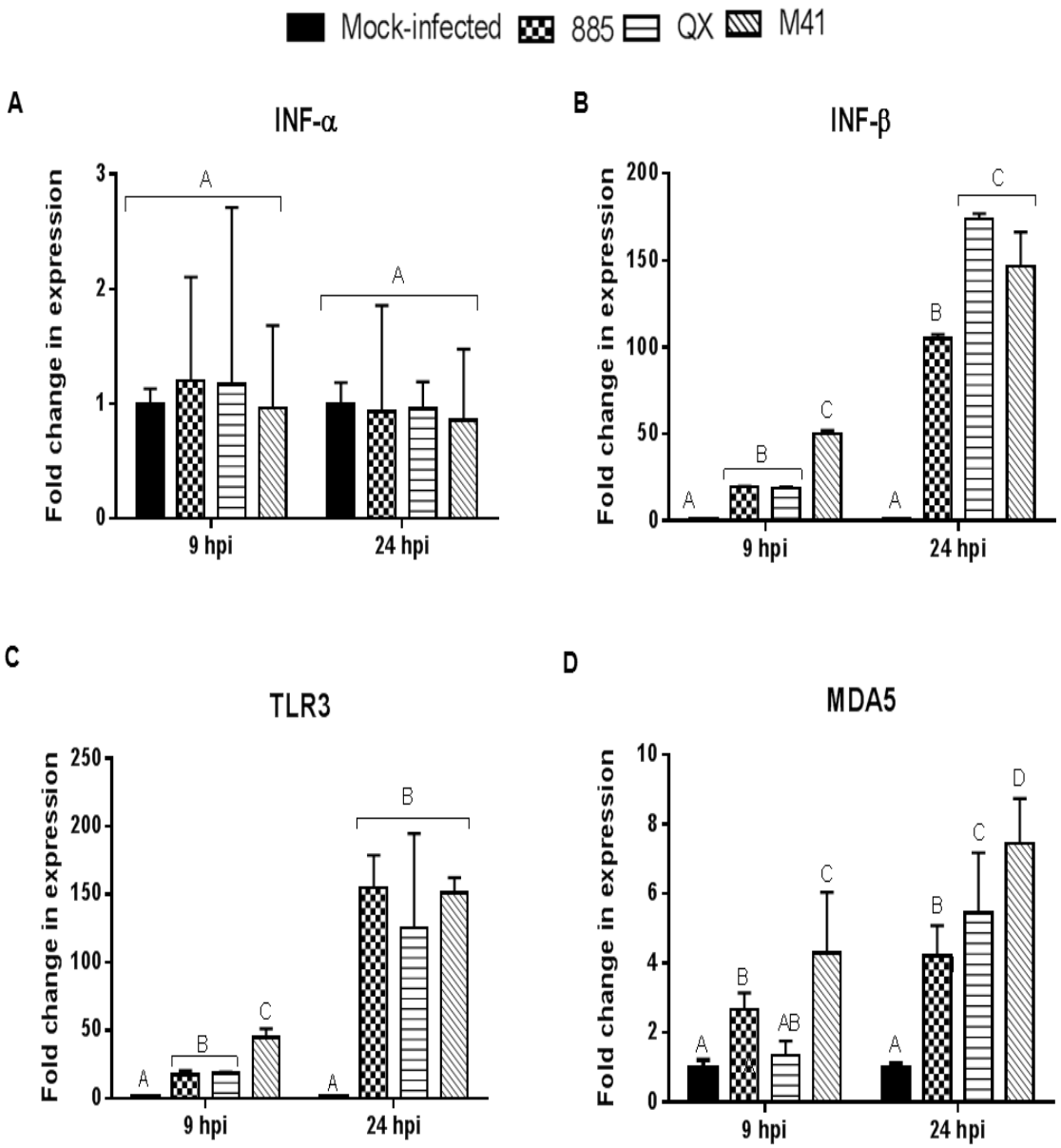


Figure 4.5. Relative mRNA expression of (A) INF-α, (B) INF-β, (C) TLR3, (D) TLR7 in TOCs infected with IBV strains 885, QX, or M41 at a dose of 4 $\log_{10}$ CD_{50} per individual ring or mock-infected. Relative mRNA expression was determined by real-time PCR normalised to 18S rRNA. Graphed values are the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mocked-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

4.4 Discussion

The use of TOCs has been shown to be the most sensitive method for the isolation of IBV from clinical material (Darbyshire *et al.*, 1975). Further, the use of TOC for investigation of virus host interactions under the controlled conditions have been exploited by many workers (Abd El Rahman, et al., 2009; Pei, et al., 2001). *In vitro* studies in TOCs are an excellent system to provide necessary data on possible adaptive mechanisms under controlled conditions. The aim of this chapter was to discover differences in early host innate immune responses and apoptosis of infected TOCs to infection by different strains of IBVs.

The quantitative assessment of ciliary activity has been used for comparing the virulence of several avian respiratory pathogens (Raj & Jones, 1996b; Stadtlander, et al., 1991). In our study, all examined IBVs showed no difference in RCA as time taken for a 50% reduction of RCA was 1.70 dpi for all viruses.

We found that IBV infection resulted in increased apoptosis in TOCs. Infection of TOCs with IBV M41 which causes mainly respiratory lesions resulted in greater apoptosis than 885 and QX. Apoptosis is a cell-death programme that is initiated in response to various extrinsic and intrinsic signals such as cellular stress, including virus infection, and plays an important role in the pathogenesis of many viruses (Wang, et al., 2007). As discussed in Chapter 3, IBVs are known to induce apoptosis in infected cells and B-cell lymphoma 2 (Bcl-2) family proteins modulate IBV-induced apoptosis (Li, et al., 2007; Liu, et al., 2001; Zhong, Liao, et al., 2012; Zhong, Tan, et al., 2012). The modulation of Bcl-2 family proteins during IBV infection has also been postulated to be under the regulation of signalling pathways such as endoplasmic reticulum (ER) stress and Mitogen-Activated Protein

Kinase/Extracellular signal-Regulated Kinase (MAPK/ERK) pathways (Fung & Liu, 2014; Zhong, Liao, et al., 2012). Further, IBV infection results in a growth-inhibitory effect by inducing cell cycle arrest at S and G_2/M phases (Li, et al., 2007) both of which results in apoptosis. Our findings suggest a strong correlation between apoptosis in TOCs to the ability of IBV strains to cause respiratory lesions in chicken. Higher apoptosis and IFN-β expression correlated with significantly lower infectious virus production from TOCs. This could be due to increased antiviral state induced by IFN-β and/or increased apoptosis. Apoptosis of cultured cells has previously been shown to be associated with reduced production of infectious influenza virus in duck cells (Kuchipudi, Dunham, et al., 2012).

Notably M41 resulted in significantly higher up-regulation of innate immune sensing genes TLR3 and MDA5 along with higher IFN-β mRNA levels in TOCs at 9 hpi than 885 or QX. Concurrent with our findings, a previous study observed a significant increase of TLR3 mRNA expression in both trachea and lungs of IBV infected chickens when compared to the uninfected controls (Kameka, et al., 2014). The end products of TLR3 signalling pathway is the production of anti-viral type I IFN-α and β (Guillot, et al., 2005). Concurrent with TLR3 activation, we found a significant up-regulation of IFN-β but not IFN-*α* mRNA expression at 9 and 24 hpi in TOCs. Several *in vitro* and *ex vivo* studies have suggested a key role of TLR3 in viral detection (Alexopoulou, et al., 2001; Bowie & Haga, 2005; Finberg & Kurt-Jones, 2004; Tabeta, et al., 2004). However, recent evidence suggests TLR3 contributes to detrimental effects of viral infections. TLR3 mediates West Nile virus entry into the brain, causing lethal encephalitis (Wang, et al., 2004) and contributes to a

detrimental inflammatory response to influenza virus infection in mice resulting in acute pneumonia (Le Goffic *et al.*, 2006).

MDA5 is IFN-inducible (Kang, et al., 2004), once IFN response is triggered, this innate antiviral loop initiates autoamplification of MDA5 until the natural inhibitor LGP2 is induced (Yoneyama, et al., 2005). In our study, MDA5 and IFN-β expression was up-regulated in TOCs at 24 hours after IBV infection compared with mock infected control. We found that M41 infection resulted in significantly higher expression of IFN-β and MDA5 than 885 or QX infection in TOCs at 24 hpi. In agreement to our findings, a more recent study has shown that *in vitro* virulent IBV infection leads to a significant induction of INFβ transcription through an MDA5-dependent activation of the IFN response (Kint, et al., 2015). In the same study, in contrast to our findings, it was also reported that TLR3 is not involved in the sensing of IBV (Kint, et al., 2015). However, higher mRNA levels of MDA5 and TLR3 correlated with apoptosis caused by IBV strains used in this study, such that M41 induced much higher levels of apoptosis in TOCs than 885 or QX. It is possible that MDA5 activation is a protective antiviral host response against the virus whereas TLR3 contributes the detrimental effects of the viral infection. The precise role of MDA5 and TLR3 in host defence against IBV infection and/or disease pathogenesis is not yet fully understood and hence, warrants further in-depth studies.

In conclusion, IBV strain M41 induced stronger innate immune response (TLR3, MDA5 and IFN-β) and apoptosis than 885 or QX in TOCs. These observed effects suggest that higher apoptosis together with elevated levels of TLR3, MDA5 and

IFN-β expression appears to be correlates to pathogenicity of IBVs in TOCs (respiratory tissue).

Chapter 5: Differential immunopathogenesis of infectious bronchitis viruses in SPF chicks

5.1 Introduction

In previous chapters, the pattern of virus infection has been studied in *vitro* or *ex vivo* to differentiate IBV strains with different tissue tropisms. It was found that transcriptional up-regulation of innate immune response genes, namely toll like receptor 3 (TLR3), melanoma differentiation associated protein 5 (MDA5) and interferon beta (IFN-β) corresponded to pathogenicity of infectious bronchitis viruses (IBVs) in kidney (CEK cells) and respiratory (TOCs) tissues. Our findings raise a tantalizing possibility of using CEKs and TOCs (*in vitro*) to rapidly predict the tissue tropism and virulence of novel IBV strains without the need of conducting more expensive and time consuming chicken *in vivo*. The primary cell culture (CEK cells) is a highly valuable tool to investigate IBV infections *in vitro*, but has some limitations as infection in chickens is a much more complex situation. Secondly*, ex vivo* (TOCs) experiments cannot include influences of immune mechanisms. This *in vivo* work was undertaken to understand the nature of the host response to different IBV strains complimenting the expanding knowledge of *in vitro* studies.

IBV strains have tropisms for different avian cell types and produces different clinical signs and pathogenic lesions according to the affected tissue (Fan *et al.*, 2012). Virulence may differ among IBV strains and, chickens also showed different susceptibilities to different IBV strains (Cavanagh, 2007; Cong, et al., 2013). After the first identification of virus in the USA in 1930s, a high diversity among isolates was reported, not only in antigenicity, but also in pathogenicity (Gelb, Wolff, et al., 1991; Lee *et al.*, 2008). The rapid detection of the variant IBVs is being done using S1 sequence analysis. However, plausible tissue tropism from this method is not always in arrangement with the actual tissue tropism (Sapats *et al.*, 1996).

Therefore, a more detailed understanding of the immunopathogenesis of IB infections is warranted.

There are few studies about the course of local mechanisms involved during IBV pathogenesis. In one study, differential immune responses of chickens have been reported to two IBV with different genotypes, KIIa and ChVI (Jang, et al., 2013). In chickens infected with KIIa genotype, simultaneous peaks in the viral copy number and upregulation of mRNA levels of pro-inflammatory cytokines (IL-6 and IL-1β) and lipopolysaccharide-induced tumor necrosis factor (TNF)-α factor (LITAF) were observed at 7 dpi in the trachea and 9 dpi in the kidney, which appeared to contribute to the scale of pathophysiological effect in the chickens. Alternatively, chickens infected with ChVI genotype showed comparatively mild upregulation in pro-inflammatory cytokines mRNA expression. In a recent report, after infection with IBV-M41 strain, an early (3 dpi) upregulation of proinflammatory cytokines IL-6 and IL-1β was reported, which coincided with the greatest scores of viral load and microscopic lesions in the trachea, suggesting a role for both of these cytokines and also virus load on the development of tracheal lesions (Okino, et al., 2014). Additionally, this report demonstrated that after infection with IBV-M41, a late (7 dpi) induction of CD8αα and Granzyme homolog A mRNA has been associated with highest scores of viral load and microscopic lesions in the trachea, suggesting a role of both these cytotoxic enzymes and virus load on the development of tracheal lesions (Okino, et al., 2014).

Pattern recognition receptors (PRRs) are present in immune cells and cells on mucosal surfaces. These cells rapidly recognize the infectious agents through PPRs such as Toll-like receptors (TLR), RIG-I like receptors (RLRs), Melanoma

differentiation-associated protein 5 (MDA5) and NOD-like receptors (NLRs). Prominent among these are TLRs. An increase in TLR3 mRNA expression has been reported in tracheal epithelial cells after intranasal inoculation with IBV-M41 strain at 3 dpi (Wang, et al., 2006) and its function in viral immunology is well established (Le Goffic, et al., 2007; Liu *et al.*, 2007). In another study, TLR2, TLR3, TLR6 and TLR7 gene expression were all significantly upregulated in the tracheal epithelial cells of 3 week old chickens immunised with attenuated IBV-Massachusetts (IBV-Mass) by intranasal inoculation (Guo, et al., 2008). MDA5 expression levels were reported to be significantly increased in chicken kidney tissue after nephropathogenic IBV infection suggesting a role of chicken MDA5 against IBV infection (Cong, et al., 2013). In a more recent study, it has been shown that *in vitro* virulent IBV infection leads to a significant induction of INFβ transcription through an MDA5-dependent activation of the IFN response (Kint, et al., 2015).

The limited information on the different immunopathogenesis of IBVs in chickens stimulated investigation of the host innate response in terms of the expression of PRRs, type I IFNs, and pro-inflammatory cytokine genes and their association with viral load profile and the course of pathogenic as well as serological changes induced by IBVs. From our studies on the comprehensive immunopathogenesis of these increasingly important IBV variants, we aim to recommend a strategy to reduce losses from IBV variants which are frequently emerging in the field.

5.2 Materials and methods

5.2.1 Virus

The viruses were propagated in SPF ECE as described in Chapter 2.3.i. To calculate the dose of infection ($10^{5.0}CD_{50}$/bird) these viruses were titrated in chicken TOCs

prepared from 19 to 20-days-old SPF chicken embryos (Chapter 2.3.iii) (Cook, et al., 1976b). Confirmation of each genotype was done by RT-PCR (Chapter 2.4.vi) followed by sequencing (Chapter 2.5.vii). The results showed that each virus had 99% identity across the part-S1 region with the published sequences. The TOC supernatant was free of NDV, AIV, avian metapneumovirus (aMPV) and infectious laryngotracheitis virus (ILTV) by RT-PCR and free of mycoplasma, bacterial or fungal contaminations.

5.2.2 SPF eggs and chicks

The SPF eggs were procured from a commercial source (Chapter 2.1.i). Fertile SPF eggs were incubated at 37°C. After checking for their fertility on day 18, eggs were transferred to hatching tray of the incubator in a static condition for hatching till day 21. Chicks were kept up to 14 days of age (doa) in an isolation unit (University of Liverpool) throughout the experiment and reared on deep litter with water and feed provided *ad libitum* as described in Chapter 2.5.ii.

5.2.3 Experimental design

One hundred SPF chicks were allocated to 4 groups (n=25). The chicks in the infected group were inoculated oculonasally with $10^{5.0}$ CD_{50}/bird of the virus and those in the control group were inoculated similarly with TOC medium to serve as uninfected controls. Clinical signs were observed daily throughout the experimental period. All euthanised and those chicks found dead were necropsied and examined for any gross lesions in trachea and kidney. At 1, 3, 7, 9 and 14 days post infection (dpi), five chicks from each group were humanely killed and trachea and kidney samples were collected. A portion was stored in RNA*Later* (Qiagen, Crawley, UK) and kept at -20°C until processing for viral load and expression of virus induced

pro-inflammatory cytokines, TLRs, and their adaptor molecules by quantitative real time RT-PCR (qRT-PCR). Trachea and kidney portions were also collected in 10% buffered formalin and examined by histopathology. At 1, 7 and 14 dpi, 5 birds in each group were bled. The blood was kept at room temperature for approximately 3 h, and the serum was separated and stored at -20°C.

5.2.4 Gross and histopathological lesions

For gross lesions, the trachea and kidney were scored individually according to the criteria described in Chapter 2.6.iii.

For histopathological examination, the upper part of the trachea and anterior kidney tissues were fixed in 10% buffered formalin, embedded in paraffin and sections were cut for hematoxylin and eosin (H&E) staining. After staining, the sections were scored individually according to the criteria described in the Chapter 2.6.ii (Chen & Itakura, 1996).

5.2.5 ELISA

Serum samples collected from all the groups at 1, 7 and 14 dpi were tested using commercial ELISA kits (IDEXX) according to the manufacturer's instructions (Chapter 2.10.i).

5.2.6 Total RNA isolation and cDNA synthesis

At each time point, total RNA was extracted from trachea and kidney samples using the RNeasy Plus Mini Kit following the manufacturer’s instructions (Chapter 2.4.i). RNA concentration was quantified by NanoDrop® ND-1000. The cDNA was generated from 1 µg of RNA using the Superscript III First-strand cDNA synthesis system with random primers as per the manufacturer’s recommendations as described in the Chapter 2.4.iii.

5.2.7 Quantification of the viral RNA

RNA was extracted from trachea and kidney samples using the RNeasy Plus Mini Kit following the manufacturer's instructions (Chapter 2.4.i). For quantification of viral RNA quantitative reverse transcription PCR (qRT-PCR) was carried out as described in Chapter 2.4.v.

5.2.8 Host gene expression analysis

QRT-PCR of cDNA samples was performed in triplicate using LightCycler 480 SYBR Green I Master mix and primers as described in the Chapter 2.4.iv. QRT-PCR data was normalized using a relative standard curve method to 18S ribosomal RNA (18SrRNA) expression (Kuchipudi, Tellabati, et al., 2012) and the data were presented as fold difference in gene expression of virus vs mock-infected samples.

5.2.9 Statistical analysis

The data from host gene expression and ELISA were analysed using one-way analysis of variance (ANOVA), followed by the post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00 software. Kruskal-Wallis test followed by Dunn's test was used for statistical analysis of the viral load by RT-qPCR and histopathological evaluation data. Differences were considered significant at $P<0.05$.

5.3 Results

5.3.1 Clinical signs and gross pathological findings

No clinical signs or mortalities were observed in the uninfected control chicks during the experiment. The chicks inoculated with 885 showed clinical signs from dpi 2 to 11. The affected chicks displayed mild tracheal rales, sneezing, coughing, head shaking and eye scratching. These clinical signs gradually disappeared by 11 dpi. The group inoculated with QX showed similar clinical signs from dpi 3 to 9. Similarly clinical signs were also observed in the chicks inoculated with M41 but, from 2 to 10 dpi. In this group, one out of 25 died at 2 dpi (4.0%).

No gross lesions were noticed in the control group. The gross lesion score seen in the infected chicks is presented in Figure 5.1. The tracheal lesions mostly comprised congestion which were started at 3 dpi in the trachea of all infected groups. No significant *(P<0.05)* differences were observed between the infected groups at any dpi (Figure 5.1 A). In all infected groups, pale kidneys with swelling with varying severity developed from 7 dpi (Figure 5.1 B). However, these lesions peaked by 9 dpi and were significantly higher in 885 inoculated group compared to QX and M41 (Figure 5.1 B).

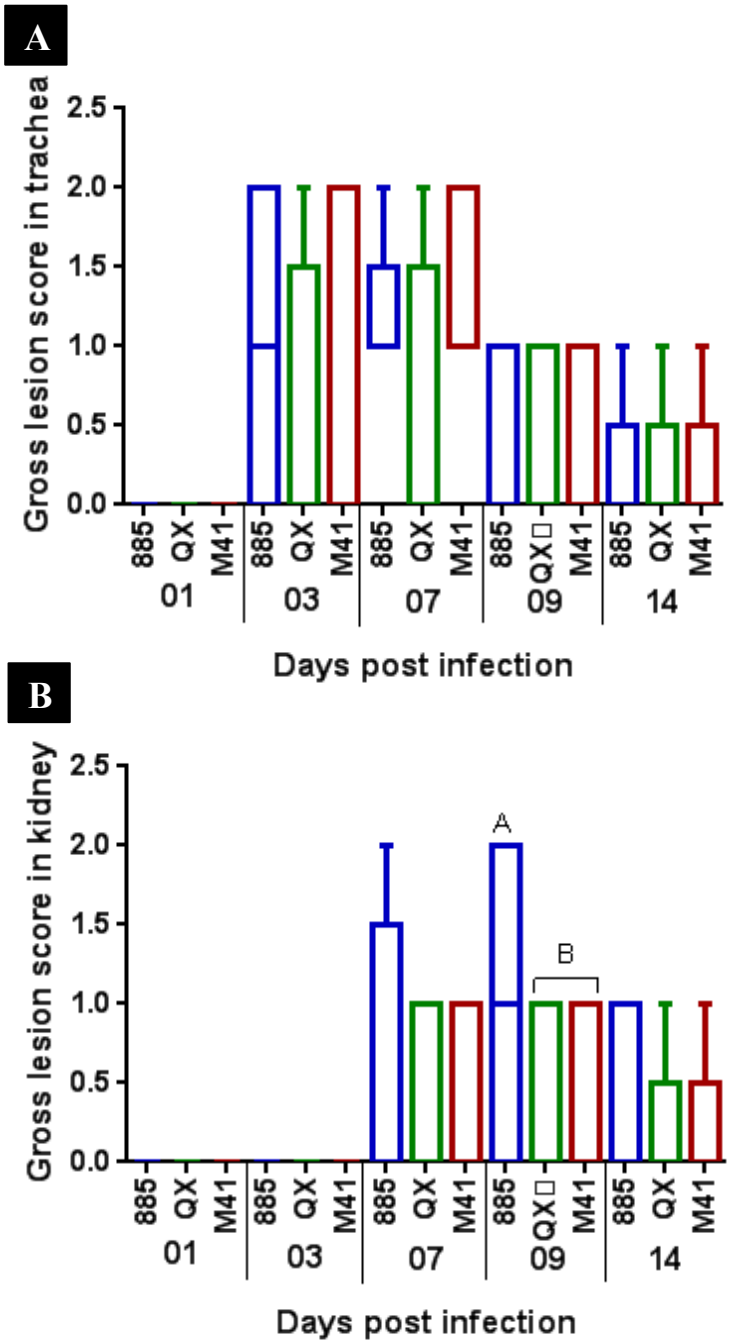


Figure 5.1. Gross lesion score in **(A)** Trachea and **(B)** Kidney samples of chicks. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and examined for gross lesions at 01, 03, 07, 09, and 14 days post infection (dpi). Data represents the median with error bars as standard error. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups at those time points.

5.3.2 Histopathology

5.3.2.1 Trachea

Normal tracheal epithelia, with healthy cilia and goblet cells, were observed in the uninfected control birds (Figure 5.2 A). In all tracheas, until 3 dpi, no statistically significant *(P<0.05)* differences were found in histopathological lesion score between the groups (Figure 5.3 A). However, pathological changes developed and peaked by 7 dpi and were significantly higher in the M41 inoculated group compared to 885 and QX (Figure 5.3 A). The most consistent lesions occurred mainly in the form of loss of cilia, decreased goblet cells, infiltration of plasma cell, heterophil infiltration, and lymphocytes (Figure 5.2 B to D).

5.3.2.2 Kidney

The kidneys of the chicks from the uninfected control group showed no obvious lesions (Figure 5.4 A). In the infected groups, the main kidney histopathological lesions consisted of interstitial lymphoid infiltration with mild lymphoid nodules (Figure 5.4 B to D), which were observed throughout the study. No significant *(P<0.05)* histological differences were found between the infected groups until 7 dpi (Figure 5.3 B). However, on 9 dpi, 885 infected group showed significantly *(P<0.05)* higher histopathological changes than those infected with QX or M41. These changes became more severe and peaked by 14 dpi where lesions in 885 infected group were significantly *(P<0.05)* greater when compared to QX or M41 infected chicks (Figure 5.3 B).

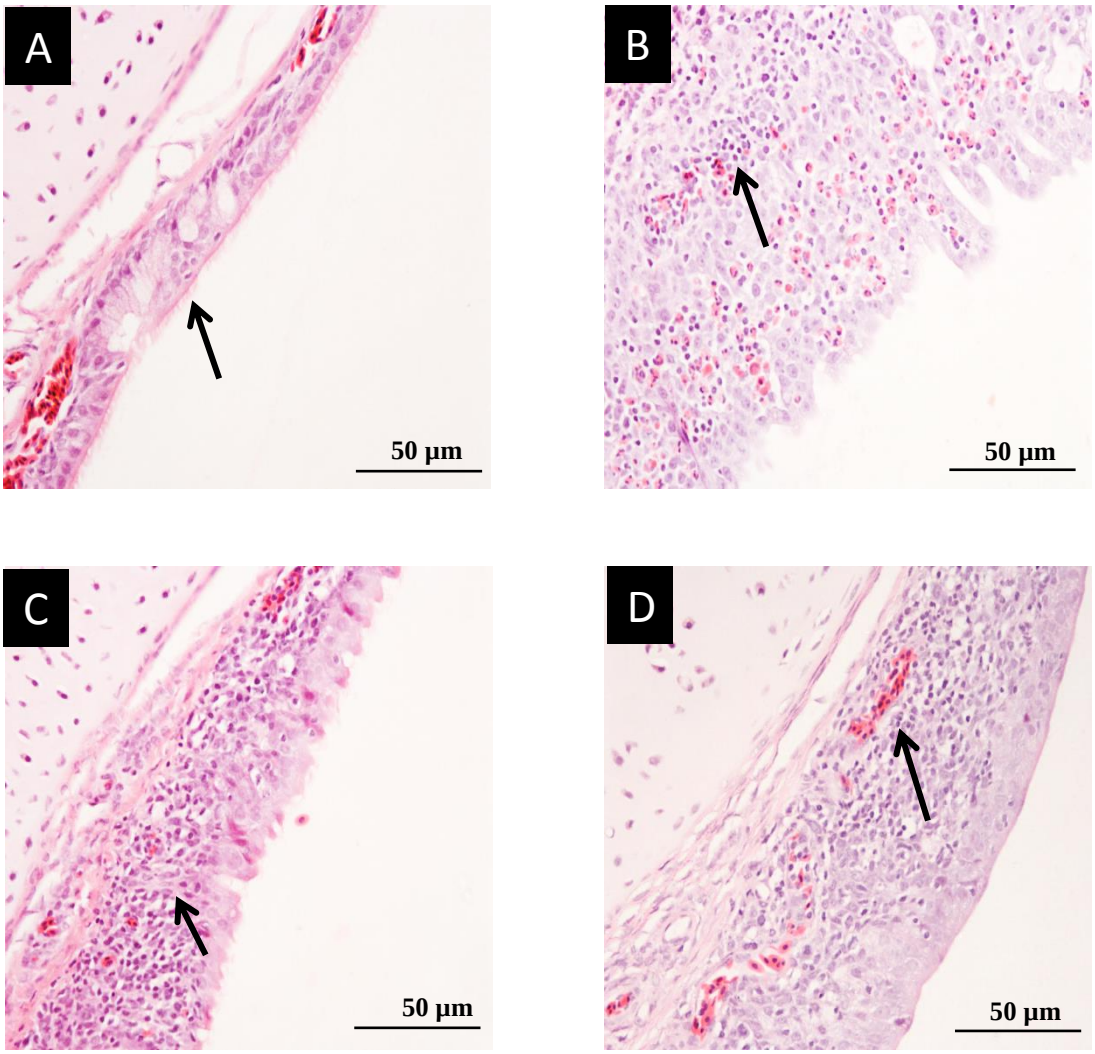


Figure 5.2. Histopathological findings in trachea samples, using Haematoxylin and Eosin (H&E) stains, at 7 days post infection (dpi) from (A) Uninfected control group showing cilia and goblet cells (B) 885 infected group showing epithelium with an infiltrate of lymphocytes, plasma cells and heterophils (C) QX infected group showing mild epithelial deciliation, with a moderate infiltrate of lymphocytes and heterophils and (D) M41 infected group showing complete epithelial deciliation with severe lymphocyte, plasma cells & heterophil infiltration and mild epithelial hyperplasia (magnification at 400x).

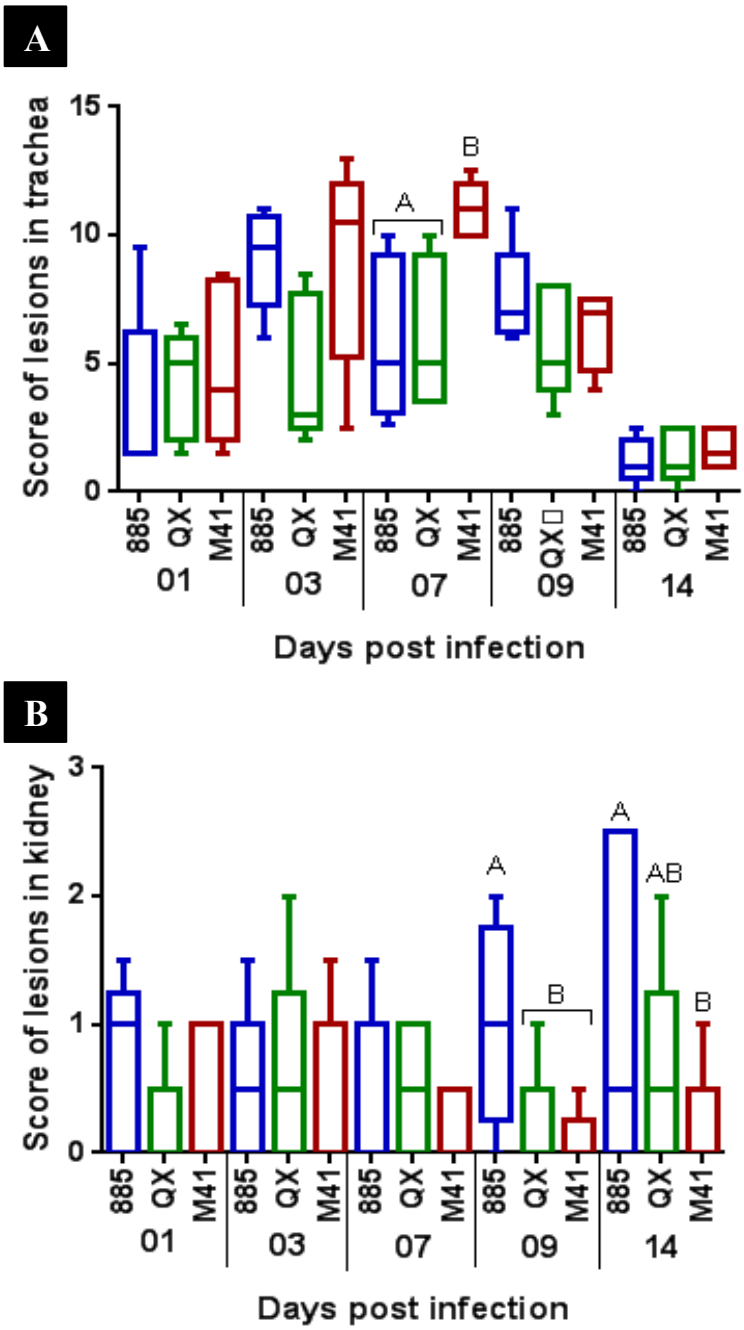


Figure 5.3. Histopathological score of lesions in **(A)** Trachea and **(B)** Kidney samples of chicks. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and processed for histopathological examination at 01, 03, 07, 09, and 14 days post infection (dpi). Data represents the median with error bars as standard error. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups at those time points.

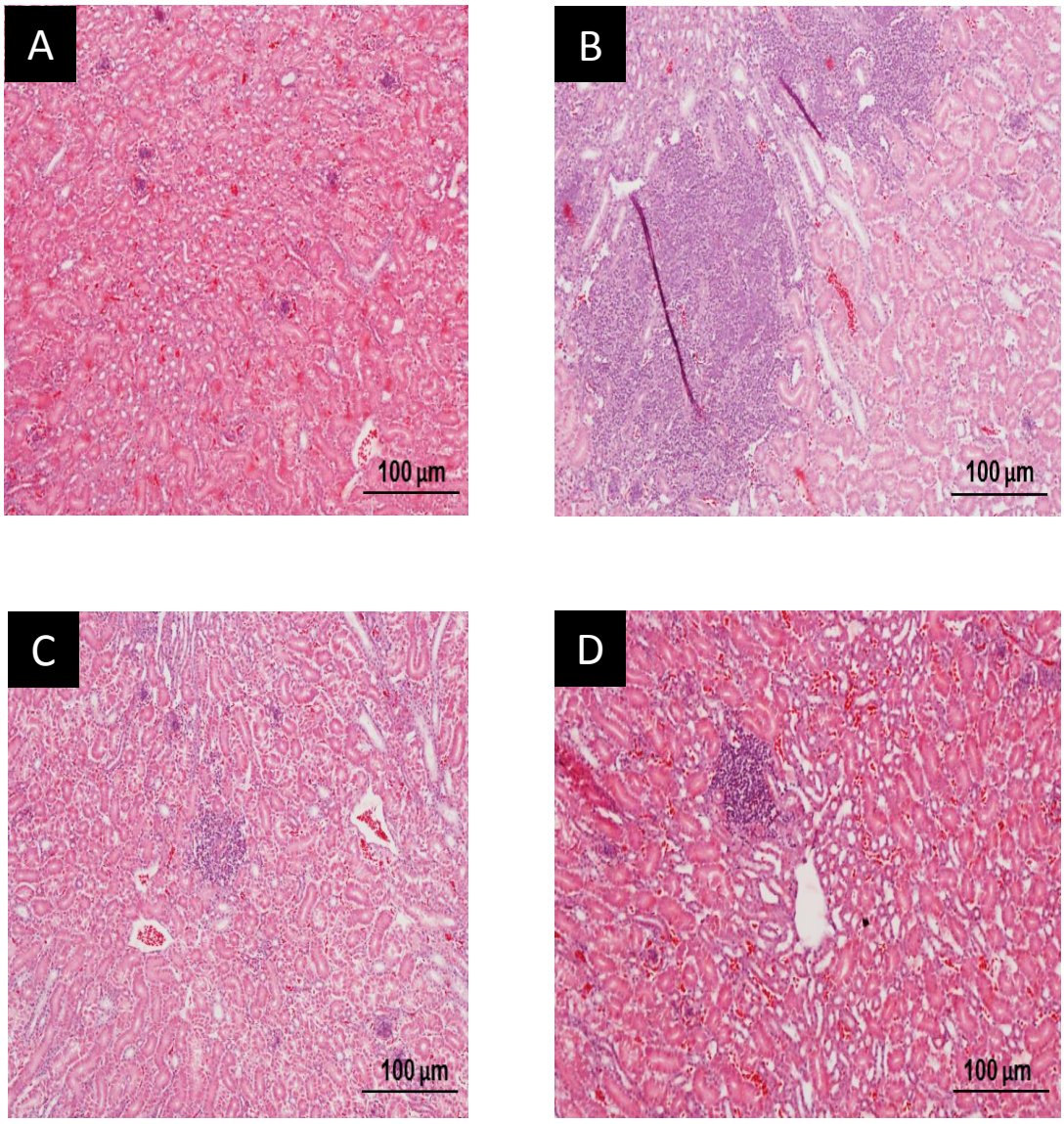


Figure 5.4. Histopathological findings in kidney samples, using Haematoxylin and Eosin (H&E) stains, at 9 days post infection (dpi) from (A) Uninfected control group (B) 885 infected group showing severe lymphoid infiltration with mild lymphoid nodules and mild heterophil interstitial infiltration (C) QX infected group showing moderate lymphocyte and heterophil interstitial infiltration (D) M41 infected group showing mild lymphocyte and heterophil interstitial infiltration (magnification at 100x).

5.3.3 ELISA

Serum samples from all chickens collected prior to infections were free of IBV antibodies (Figure 5.5). At 7 dpi, all chicks inoculated with different IBV strains showed a positive IBV antibody response without any significant (*P<0.05*) difference between the groups, whereas chicks in the uninfected control group showed a negative serum antibody response. Subsequently, the antibody levels in all the three groups peaked at 14 dpi whereas, M41 infected group showed significantly *(P<0.05)* higher antibody response followed by QX than 885 infected chicks (Figure 5.6).

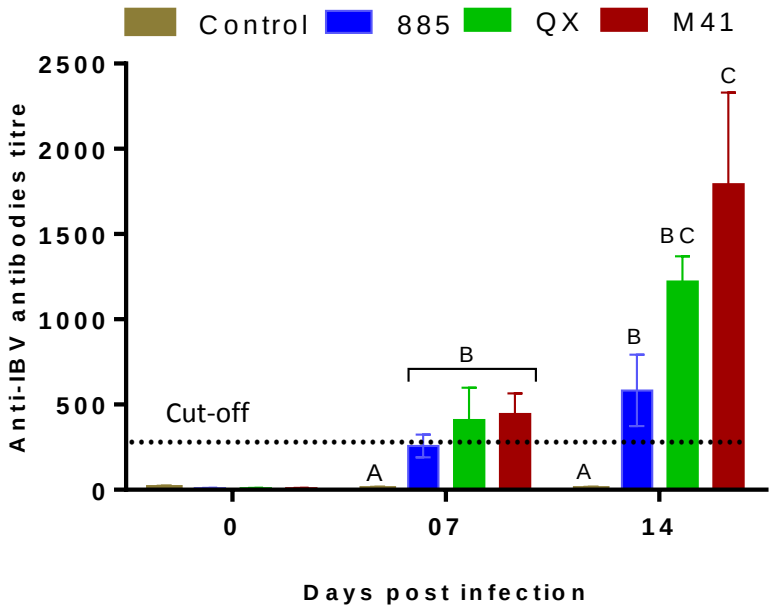


Figure 5.5. Infectious bronchitis virus (IBV) antibody titres of the different groups (n=5/group) infected with IBV strains 885, QX, M41 or uninfected control at a dose of $10^{5.0}$ CD_{50}/bird. Data are expressed as mean values ± SEM (n=5). Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

5.3.4 Viral RNA quantification

Viral RNA load in trachea and kidney was measured using the qRT-PCR (Figure 5.6).

5.3.4.1 Trachea

Viral RNA loads in the tracheas of birds in the uninfected control group were below the detection limit on all days post infection (Figure 5.6 A). At 1 dpi, all the tracheal samples collected from the infected groups were positive without any significant difference between the groups (Figure 5.6 A). The viral RNA load in all the infected groups peaked at 7 dpi and there was no significant (*P<0.05*) differences between these groups. Thereafter, viral RNA load decreased in all the infected groups and at 14 dpi, nearly reached basal levels.

5.3.4.2 Kidney

The viral RNA load in the kidneys of birds from the uninfected control group on all days post infection were below the detection limit and also from infected groups until 3 dpi (Figure 5.6 B). At 7 dpi, all the kidneys samples from the infected groups were positive without any significant difference between the groups. However, at 9 dpi, viral RNA load was significantly higher (*P<0.05*) in the 885 infected group compared to those infected with QX or M41. Viral RNA loads in kidneys from all the infected groups peaked at 14 dpi however; it was significantly (*P<0.05*) higher in group infected with 885 followed by those infected with QX and M41 (Figure 5.6 B).

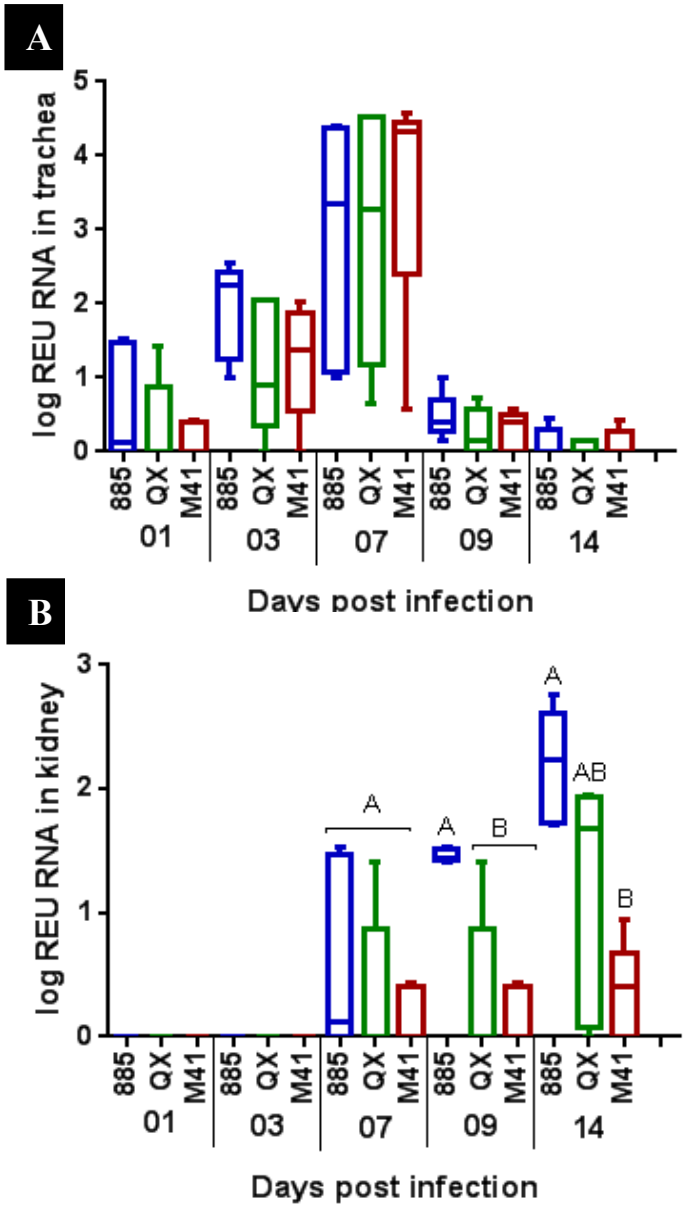


Figure 5.6. Quantification of viral RNA, expressed as a log relative equivalent units (REU) of RNA in (A) Trachea and (B) Kidney samples of chicken. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and analysed for quantification of viral RNA at 01, 03, 07, 09, and 14 days post infection (dpi). Data represents the median with error bars as standard error. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P*<0.05*)* different between the groups at those time points.

5.3.5 Host gene expression analysis

5.3.5.1 Relative IFN-α and IFN-β mRNA expression

5.3.5.1.1 Trachea

The mRNA expression data for IFN-α and IFN-β in the trachea are illustrated in Figure 5.7 A & B, respectively. No significant change in the IFN-α mRNA expression levels at any dpi was noticed in the infected groups compared with uninfected control group (Figure 5.7 A). Notably, at 1 dpi, tracheal samples of chicks infected with M41 resulted in significantly (*P<0.05*) greater IFN-β mRNA expression than those infected with 885 or QX (Figure 5.7 B). Conversely, on subsequent dpi no significant (*P<0.05*) changes in level of IFN-β mRNA expression were detected in any infected group compared with uninfected control group.

5.3.5.1.2 Kidney

The mRNA expression data for IFN-α and IFN-β in the kidney are illustrated in Figure 8 A & B, respectively. No significant (*P<0.05*) differences in the IFN-α expression levels at any dpi was noticed in any infected group compared with uninfected control group. At 1 dpi, IFN-β mRNA expression in kidney samples of chicks infected with 885 was up-regulated (*P<0.05*) whereas, it was down-regulated (*P<0.05*) in M41 infected group compared with uninfected control group. However, at 3 dpi it was significantly (*P<0.05*) higher in the group infected with 885 compared to those infected with QX. At this time point, no significant change was noticed in M41 infected group compared with uninfected control (Figure 5.8 B). No significant changes in level of IFN-β expression was observed in any infected group at the other time points (7, 9 or 14 dpi).

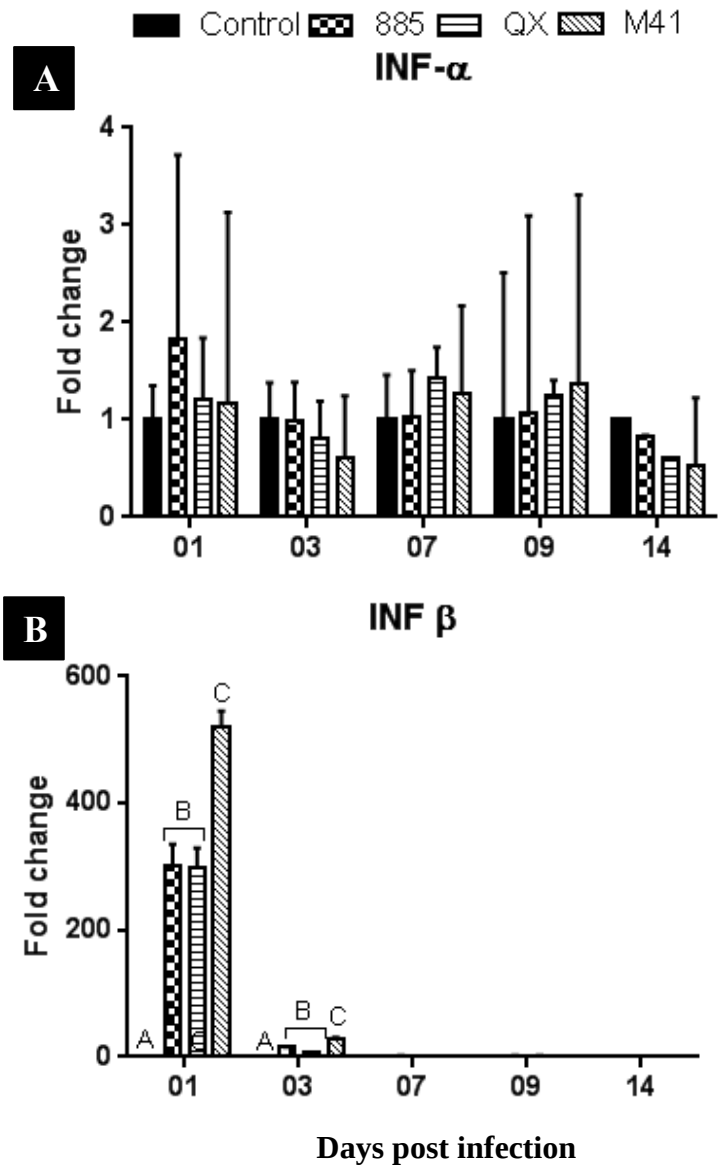


Figure 5.7. Relative Type I interferon (IFN) (A) IFN-α and (B) IFN-β mRNA expression in IBV infected trachea of chicks. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)* and all other values were not significantly *(P<0.05)* different between the groups.

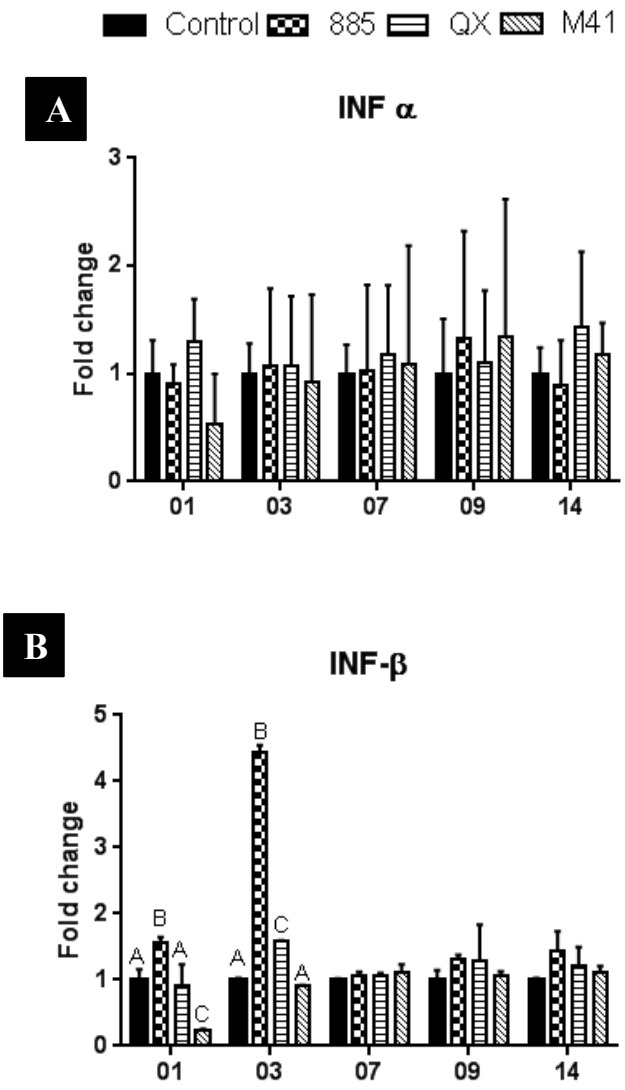


Figure 5.8. Relative Type I interferon (IFN) (A) IFN-α and (B) IFN-β mRNA expression in IBV infected kidney of chicks. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)* and all other values were not significantly different between the groups at those time points.

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

5.3.5.2.1 Trachea

The mRNA expression of TLR3 and MDA5 in the tracheal samples from chicks infected with IBV strains 885, QX, M41 or uninfected control group are illustrated in Figure 5.9 A & B, respectively. It was observed that expression of TLR3 (Figure 5.9 A) in all the infected groups were significantly (*P<0.05*) up-regulated at 1 dpi compared to the uninfected control group. At this time point, M41 infected group showed significantly *(P<0.05)* higher level of expression as compared to 885 or QX. However, at 3 dpi, infection of IBV strains M41 and QX resulted in significantly greater TLR3 mRNA expression than 885 infection in the trachea (Figure 5.9 A). No significant (*P<0.05*) changes in TLR3 mRNA expression levels were detected in any infected group compared with uninfected control group on subsequent dpi. Similarly, infection with IBV strains M41 and QX resulted in significantly (*P<0.05*) greater MDA5 expression than 885 infection in the trachea at 1 dpi (Figure 5.9 B). No significant (*P<0.05*) changes in level of MDA5 expression were observed in any infected group at the other time points (3, 7, 9 or 14 dpi).

5.3.5.2.2 Kidney

The mRNA expression data for TLR3 and MDA5 in the kidney are illustrated in Figure 5.10 A & B, respectively. In contrary to the mRNA expression of these two genes in tracheal samples infected with IBVs, at 3 dpi, 885 infection resulted in significantly (*P<0.05*) higher mRNA expression in the kidneys of TLR3 (Figure 5.10 A) followed by QX and M41 infection. Notably, no significant (*P<0.05*) changes in level of TLR3 mRNA expression were observed in any infected group at the other time points (1, 7, 9 or 14 dpi) compared with the uninfected control group. At 3 dpi,

MDA5 mRNA expression (Figure 5.10 B) in kidney samples of chicks infected with 885 was up-regulated and was significantly (*P<0.05*) higher compared to those infected with QX. However, no significant (*P<0.05*) change was observed in group infected with M41 compared with the uninfected control group. At 7 dpi, MDA5 mRNA expression level in all infected groups was significantly (*P<0.05*) up-regulated compared with the uninfected control group but, without any significant (*P<0.05*) difference between the infected groups.

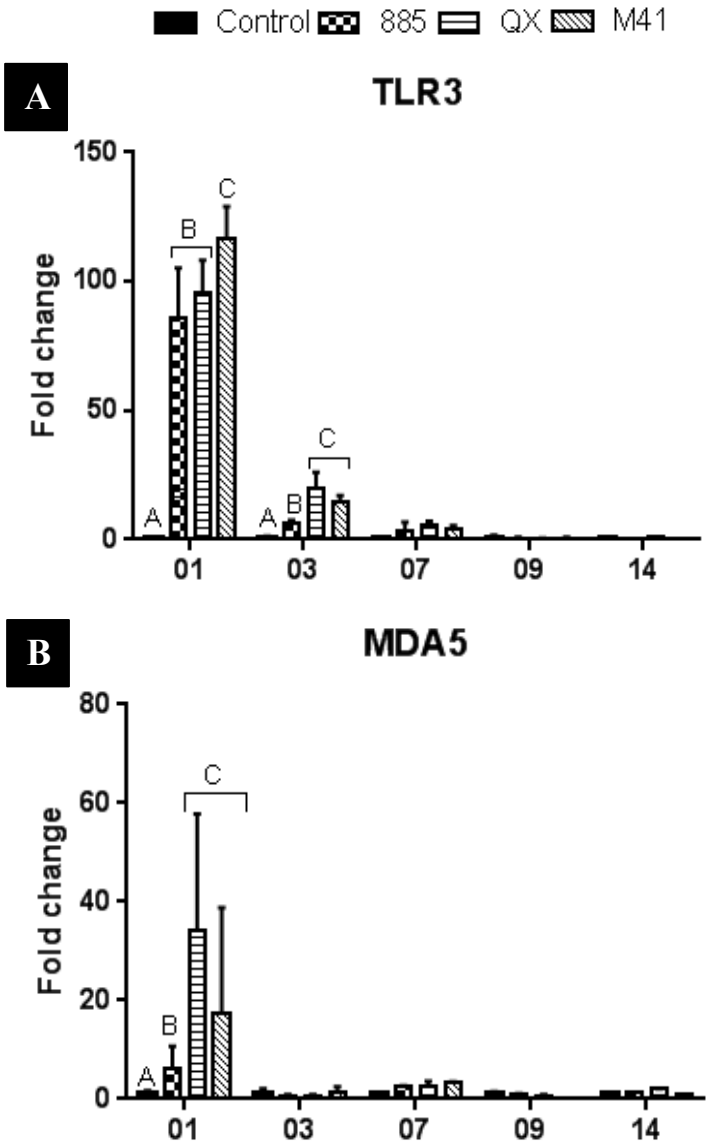


Figure 5.9. Transcriptional regulation of innate viral sensing molecules (A) TLR3 and (B) MDA5 in chicken trachea. Birds (n=5/group) were infected with IBV strains 885, QX, or M4 at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

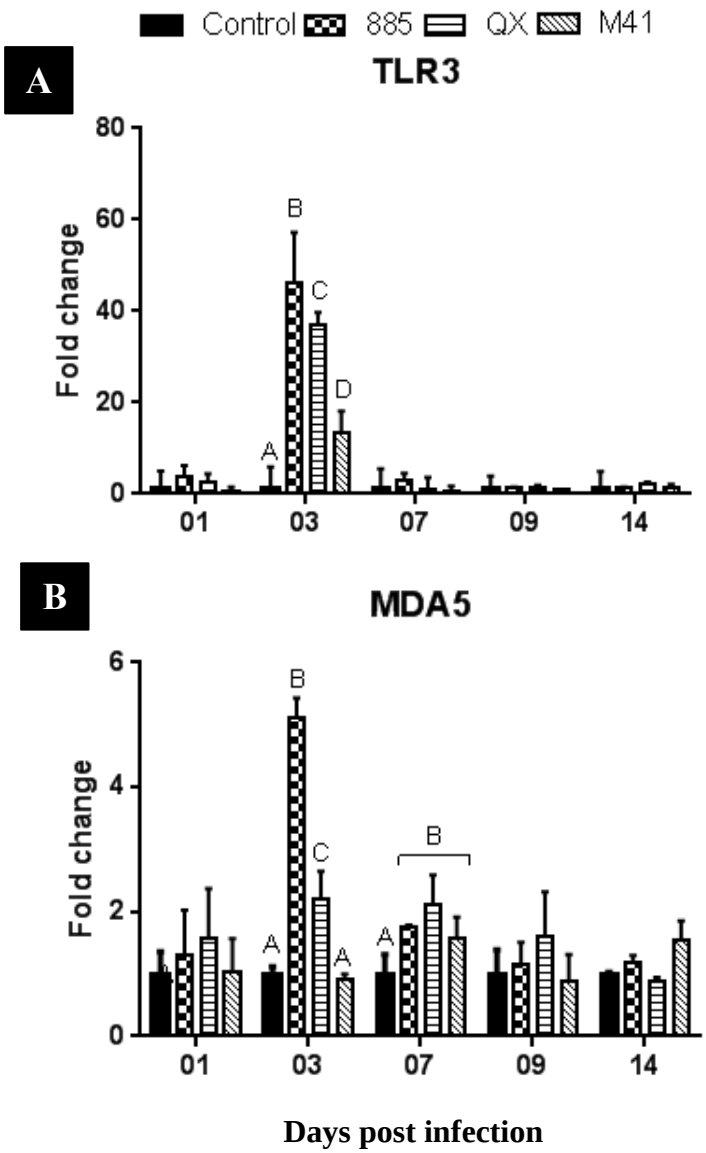


Figure 5.10. Transcriptional regulation of innate viral sensing molecules (A**)** TLR3 and (B) MDA5 in chicken kidney. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

5.3.5.3 Transcription profile of proinflammatory cytokines in the trachea and kidney

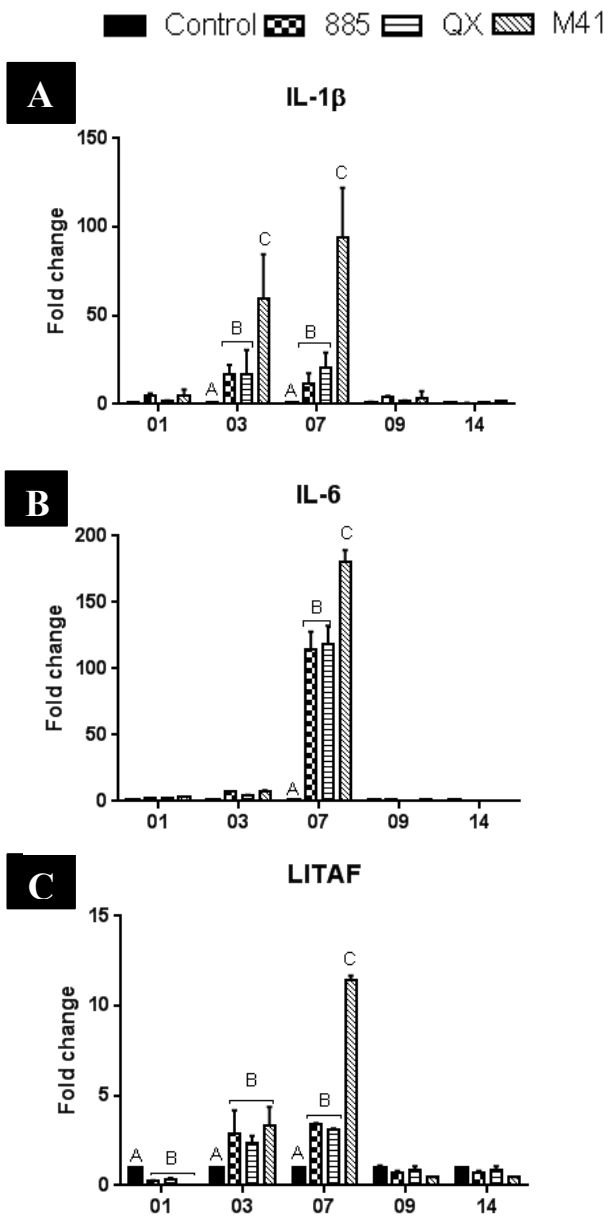
5.3.5.3.1 Trachea

The changes in levels of 3 pro-inflammatory cytokines in the trachea are illustrated in Figure 5.11. The results indicate that, the level of IL-1β mRNA expression peaked at 7 dpi in all infected groups compared with uninfected control, with mRNA expression being significantly *(P<0.05)* higher at 3 and 7 dpi in chicks infected with M41 as compared with 885 or QX infection. Figure 11B shows the expression of IL-6 wherein, at 7 dpi, a significant *(P<0.05)* increases in the expression was noticed in chicks infected with M41 as compared to 885 or QX. No significant *(P<0.05)* changes in level of IL-6 expression was seen in any infected group at any other time points compared with the uninfected control group. At 1 dpi, LITAF transcription levels in all the infected groups was significantly *(P<0.05)* down-regulated compared with the uninfected control group. However, at 3 dpi, LITAF mRNA expression level in all infected groups was significantly (*P<0.05*) up-regulated compared with uninfected control group but, without any significant (*P<0.05*) difference between the infected groups. LITAF mRNA expression was seen to have the same pattern as the IL-6 measured in trachea, as at 7 dpi, M41 infected group showed significantly *(P<0.05)* higher level of expression as compared to 885 or QX (Figure 5.11 C).

5.3.5.3.2 Kidney

Conversely, in the kidney samples, IL-1β mRNA expression level expression peaked at 9 dpi in all infected groups and was significantly *(P<0.05)* up-regulated in 885 infected group followed by QX and M41 compared to the uninfected control group

(Figure 5.12 A). At 7 and 14 dpi, IL-1β mRNA expression in all infected groups was significantly (*P<0.05*) up-regulated compared with uninfected control group but, without any significant difference between them. The mRNA expression of IL-6 in kidneys from all the infected groups peaked at 9 dpi (Figure 5.12 B) and it was significantly (*P<0.05*) up-regulated compared with the uninfected control group but, without any significant (*P<0.05*) difference between them. However, IL-6 mRNA expression level was significantly *(P<0.05)* up-regulated at 14 dpi in 885 infected group followed by QX and M41 compared to uninfected control group (Figure 5.12 B). The LITAF mRNA expression level was significantly *(P<0.05)* up-regulated in all infected groups compared with uninfected control at 7 dpi and it was significantly *(P<0.05)* higher in group infected with 885 and QX as compared to M41 (Figure 12 C). However, at 9 dpi, a significant *(P<0.05)* increases in the LITAF mRNA expression was noticed in chicks infected with 885 as compared to QX or M41. Notably, we found that 885 infection resulted in significantly (*P<0.05*) higher mRNA expression of LITAF (Figure 5.12 C) followed by QX and M41 infection in kidneys at 14 dpi.



Days post infection

Figure 5.11. Transcription profile of proinflammatory cytokines (A) IL-1β, (B) IL- 6 and (C) LITAF in IBV infected chicken trachea. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 1 at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

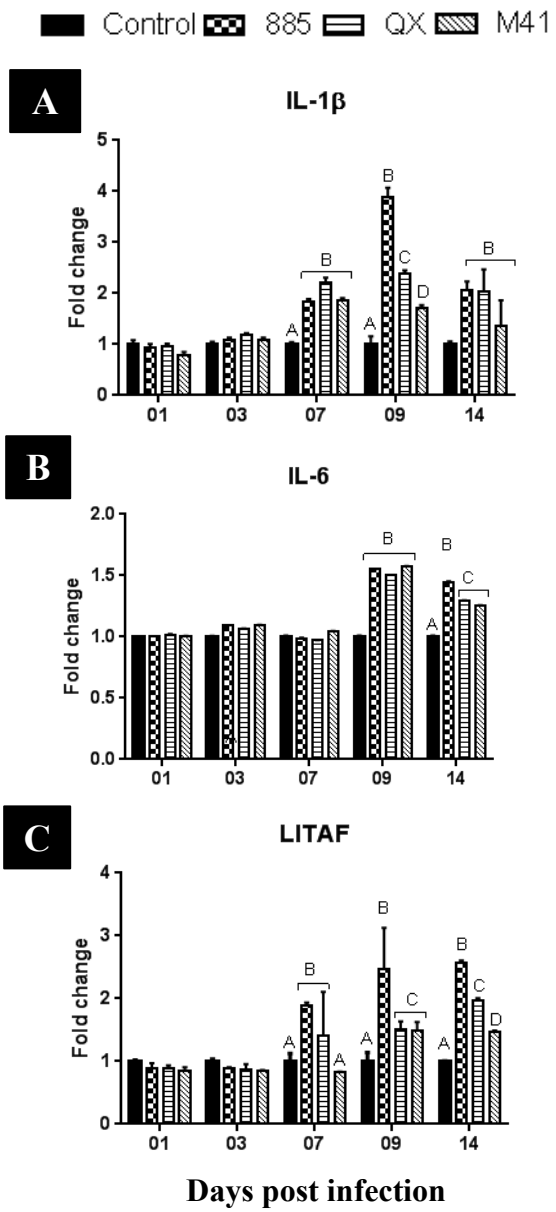


Figure 5.12. Transcription profile of proinflammatory cytokines (A) IL-1β, (B) IL-6 and (C) LITAF in IBV infected chicken kidney. Birds (n=5/group) were infected with IBV strains 885, QX, or M41 at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

5.4 Discussion

IBV causes an acute and highly contagious disease in chicks, primarily targeting the trachea and kidney. The investigation of the course of local immunological and cytokine induced mechanisms involved in IBV pathogenesis will help to elucidate the IBV-host interaction and the pathogenic mechanisms of IBV. These pathogenic mechanisms may differ among IBV strains particularly regarding the kinetics, magnitude, and duration of local inflammatory responses and the immune mechanisms in the target organs. Our findings from *in vitro* study showed the possibility of using CEKs and TOCs to rapidly predict the tissue tropism and virulence of novel IBV strains. The main advantage of using *in vitro* model is that explants from the same animals can be used to compare different viral strains and for corroborating the *in vitro* results. In this study, we investigated the differential immunopathogeneses in chickens infected with three IBV strains used in the *in vitro* studies. We noted that infected chicks displayed mild clinical signs like tracheal rales, sneezing, coughing, head shaking and eye scratching similar to the signs which have been observed following infection with other virulent IBVs (Ambali & Jones, 1990; Butcher *et al.*, 1990; Dolz, et al., 2012). However, the duration of these clinical signs differed between IBV infected groups indicating differences in the susceptibility of chicks to infection by these three IBV strains. In addition, the infection of chicks by IBV strains M41 and QX resulted in significantly higher antibody levels in serum at 14 dpi than 885 though all groups of chicks received the same amount of virus. Hence, we examined the differential modulation of PRRs (TLR and MDA5), type I IFNs, & pro-inflammatory cytokine

mRNA expression in chicks and its association with viral load profile and the course of histopathological changes induced by different IBVs.

In the present study, each strain showed obvious histopathological changes in trachea and kidney similar to reported in previous experiments (Chousalkar *et al.*, 2007; Kotani *et al.*, 2000). Based on analysis of the three strains separately, the pathological changes in the trachea peaked by 7 dpi in all groups but were significantly higher in the group infected with M41 compared to 885 and QX. A similar trend was found for viral load in the trachea, though no significant differences were observed between the groups. At 7 dpi, peak values for histopathological changes and viral load were found to be associated with peaks of expression of inflammatory cytokines, such as IL-6 and IL-1β, and higher LITAF mRNA expression values. Interestingly, at 7 dpi in trachea mRNA expression values for these inflammatory cytokines were significantly higher in M41 compared to 885 and QX. In kidneys, temporal associations between peak histopathological lesion scores and peak viral load were observed. However, the kinetics of peak values of histopathological changes and viral load were not in accordance with those of the inflammatory cytokines. In contrast to trachea, the higher values of histopathology scores, viral load and the expression of inflammatory cytokines were found in 885 and QX infected group as compared to M41. The role of the proinflammatory cytokine, IL-6 has been investigated for its contribution to nephritis in two genetic lines of chickens (Asif, et al., 2007). In this study, IL-6 transcripts were found to be directly related to renal lesions in chicks challenged with a nephropathogenic strain of IBV. In the more susceptible S-line chicks, these IL-6 levels were 20 times higher than those in the more resistant HWL chicks. Concurrent with our findings,

differential immune responses of chicks to two IBV with different genotypes, KIIa and ChVI has also been reported (Jang, et al., 2013). In chicks infected with KIIa genotype, simultaneous peaks in the histopathological lesions, viral copy number and upregulation of mRNA levels of pro-inflammatory cytokines (IL-6 and IL-1β) and LITAF were observed in the trachea at 7 dpi and in the kidney at 9 dpi which appeared to have a detrimental effect on these tissues. This has also been supported by a more recent study (Okino, et al., 2014), in which upregulation of expression of proinflammatory cytokines such as IL-6 and IL-1β, induced by the M41 strain of IBV, was found to be partially associated with the pathological alterations and/or viral load in trachea of non-immune challenged chicks. Interestingly, in our study of mRNA expression values for these inflammatory cytokines, levels were significantly higher in M41 infected chicks compared to 885 and QX in trachea. In contrast, levels were higher in 885 and QX infected group as compared to M41 in the kidney, suggesting a role of these cytokines in the development of different histopathological lesions in these organs caused by different IBVs.

TLR3 is known for recognition of RNA virus encoded pathogen associated molecular patterns (PAMPs) and its function in viral immunology is well established (Akira, 2001; Le Goffic, et al., 2007). It was observed that IBV strains M41 and QX resulted in significantly higher up-regulation of innate immune sensing gene TLR3 levels in tracheas at 3 dpi when compared to 885 infections. In contrast, upregulation was significantly greater for these genes in the 885 infected kidneys than those infected with QX or M41 at 1 dpi. Parallel with our findings, previous studies have also reported a significant increase of TLR3 mRNA expression in the trachea of IBV

infected chicks when compared to the uninfected controls (Kameka, et al., 2014; Wang, et al., 2006). TLR3 mediates West Nile virus entry into the brain, causing lethal encephalitis (Wang, et al., 2004) and contributes to a harmful inflammatory response to influenza virus infection in mice resulting in acute pneumonia (Le Goffic, et al., 2007).

In this study, at 1 dpi MDA5 mRNA expression values were significantly higher in M41 or QX infected tracheas compared to those with 885 infection. In contrast, expression levels were significantly upregulated in 885 infected kidneys compared to those infected with QX or M41 at 3 dpi. In agreement with this, in our *in vitro* studies (Chapters 3 and 4) we found that compared to M41, 885 and QX, caused greater induction of TLR3 and MDA5 in CEK cells. In contrast, M41 infection caused greater expression of these genes than 885 or QX in TOCs. MDA5 mRNA expression levels were reported to be significantly increased in chicken kidney tissue after nephropathogenic IBV infection, suggesting a role of chicken MDA5 against IBV infection (Cong, et al., 2013). This has further been supported by a more recent study in which it has been shown that *in vitro* virulent IBV infection leads to a significant induction of INFβ transcription through an MDA5-dependent activation of the IFN response (Kint, et al., 2015). Altogether, we observed that mRNA levels of TLR3 and MDA5 correlated with the pattern of histopathological lesion scores in tracheas and kidneys produced by IBV strains used in this study.

Innate immune responses comprised a network of varied antimicrobial mechanisms, of which the type I IFN response is an essential defense mechanism against viruses. Concurrent with TLR3 and MDA5 activation, we found a significant up-regulation of IFNβ, but not IFNα mRNA expression, after IBV infection at 1 dpi in

the trachea and 3 dpi in the kidney. Many previous studies claim that the activation of the TLR3 pathway works towards an up-regulation of INF-β production in chickens (Kameka, et al., 2014; Karpala *et al.*, 2008; Parvizi *et al.*, 2012). The kinetics of IFN response observed in our study is in line with the previous studies; however a more recent study showed that MDA5, but not TLR3, is involved in detection of IBV (Kint, et al., 2015). Though, higher mRNA levels of MDA5 and TLR3 correlated with the most pathogenic strains of IBV in trachea and kidney. We also noted that tracheal samples from chicks infected with M41 resulted in significantly greater IFN-β expression when compared to those infected with 885 or QX. In contrast, 885 and QX infection caused higher induction of this gene than M41 in the kidney. A similar trend of IFN-β expression was noted in our *in vitro* study (Chapter 3 and 4) wherein, up-regulation was observed at 9 and 24h after IBV infection in CEK cells and TOCs, respectively. Early studies on gamma coronaviruses in chickens also suggested that IBV-induced IFN production is variable and dependent on both the virus strain and cell type (Holmes & Darbyshire, 1978; Otsuki *et al.*, 1988; Otsuki, et al., 1987).

In this study, we reported that the histopathological changes, pro-inflammatory and innate immune gene responses could be induced to a diverse range depending on the strain of IBV. In conclusion, our results indicate that higher upregulation of expression of proinflammatory cytokines (such as IL-6 and IL-1β) and LITAF are induced by the M41 strain in the trachea and 885 and QX in the kidney, which are partially implicated in the respiratory and renal lesions caused by these viruses, respectively. In addition, increased levels of TLR3, MDA5 and IFN-β expression correlated to the severity of pathogenicity of IBVs in the trachea and kidney.

Chapter 6: New insights into *in vitro* and *in vivo* immunopathogenesis of infectious bronchitis virus 793B serotype

6.1 Introduction

Infectious bronchitis virus (IBV), the prototype of the family *Coronaviridae* of the order *Nidovirales*, is an important pathogen in chickens. The M41 and 793B are among the most important and prevalent serotypes of IBV worldwide (Jackwood, 2012). The first known strain of the 793B serotype, also known as 4/91 (Parsons *et al.*, 1992) and CR88, was isolated in France in 1985 (Picault *et al.*, 1986). This serotype may have entered the UK in the winter of 1990/91, when it was sometimes associated with mortality in breeding hens and deep pectoral muscle myopathy (Gough *et al.*, 1992; Parsons, et al., 1992). Afterward, it was discovered that this serotype had been present in most European countries (de Wit, et al., 2011). In Asia, the first time the 793B serotype was recorded was in China in 2003 where it was named Taian-03, (accession number AY837465). Presently, 793B type viruses have been detected in several Asian countries, including Japan (Mase *et al.*, 2008), India (Sumi *et al.*, 2012) and Iran (Bijanzad *et al.*, 2013). A more recent study has reported the presence of several variant field viruses from many Middle East countries from 2009-2014 (Ganapathy *et al.*, 2015). Among these, 793B was the dominant genotype detected by this study.

Vaccination is generally considered essential for the protection of commercial chickens from virulent IBVs (Jackwood & de Wit, 2013). Massachusetts (M41) and H120 strains, in combination with 793B, are the most commonly used in many parts of the world (Jackwood & de Wit, 2013). Such vaccines have been proven to confer protection against a wide range of IBV strains, including homologous and non-homologous IBV genotype/serotypes (Awad *et al.*, 2015; Cook JKA, 1999; Jackwood & de Wit, 2013). However, for almost two decades, 793B has been

associated with disease and for most of that period one or more live 793B vaccines have been in use (Cook *et al.*, 1996; Martin *et al.*, 2014). Of late, in a study on genotypes of IBVs circulating in the Middle East between 2009 and 2014, the 793B field strain has been shown to be clustering distinctly compared to strains from earlier years (Ganapathy, et al., 2015). The alterations in translated amino acid sequences were reported to be minimal but still may have played an important role in the persistence of this virus, despite the use of live 793B vaccines (Ganapathy, et al., 2015).

Based on the above facts, persistence of 793B strains is of concern for effective control of diseases caused by IBV. There are limited reports on pathogenesis and host immune responses to 793B (Bijanzad, et al., 2013; Boroomand *et al.*, 2012; Raj & Jones, 1996a). As IBV 793B infections continue to be a major threat to poultry health in many parts of the world, better understanding of immunopathogenesis of the virus need to be developed to control the infection adequately. To further examine on the interaction between the 793B and host, a series of *in vitro* and *in vivo* examinations were conducted similar to one in Chapter 3, 4 and 5.

6.2 Materials and methods

6.2.1 Cell and organ cultures

Monolayers of primary chicken kidney (CEK) cells were prepared from kidneys of specific-pathogen-free (SPF) chicken embryos after 18 day incubation as described in Chapter 2.1.ii. Chick tracheal organ cultures (TOCs) were prepared from 18-day-old SPF ECE (Chapter 2.1.iii).

6.2.2 SPF chicks

The SPF eggs were procured from a commercial source (Chapter 2.1.i) and hatched in our facility as described in Chapter 5. Chicks were kept up to 14 days of age (doa) in an isolation unit (University of Liverpool) throughout the experiment and reared on deep litter with water and feed provided *ad libitum* as described in Chapter 2.5.ii.

6.2.3 Virus

The IB virus was isolated from caecal tonsils of a laying flock that had suffered a drop in egg production. The virus stock was propagated in chicken TOCs prepared from 19 to 20-day old SPF ECE (Chapter 2.3.i). Confirmation of genotype was done by RT-PCR (Chapter 2.4.vi) followed by sequencing (Chapter 2.5.vii). The results showed that the virus had 99% identity across the S1 region with published sequences. The TOC supernatant was free of, NDV, AIV, avian metapneumovirus (aMPV) and infectious laryngotracheitis virus (ILTV) by RT-PCR, and free of bacterial or fungal contaminations.

6.2.4 Virus titration

For CEK cell infection study, the virus was titrated on CEK cells using an immune cytochemical focus assay described previously in Chapter 2.3.iv and expressed as MOI (Nelli, et al., 2012). For infection of TOC and chicks, virus was titrated in TOC and expressed in median ciliostatic doses (CD_{50})/ml as discussed in detail in Chapter 2.3.iii (Cook, et al., 1976b).

6.2.5 Experimental design

6.2.5.1 Virus infection in cell and organ cultures

Confluent monolayer of CEK cells was washed three times with PBS, and then infected with 793B strain of IBV at a multiplicity of infection (MOI) 1.0 or mock infected with cell culture medium (Appendix I) as described in Chapter 3.3.3. Cells were analysed at 24 and 48h post-infection (hpi) to determine the effect of virus infection on cell metabolic activity, induction of apoptosis and cell culture supernatant to titrate infectious virus in TOC expressed as CD_{50}/ml (Cook, et al., 1976b).

TOCs were infected with 793B strain using a dose of 4 $\log_{10}$ CD_{50} per individual ring or mock-infected with TOC medium (Appendix I) without virus as detailed in Chapter 4.3.3. Samples of TOC supernatant or complete rings were collected at 24 and 48 hpi. TOC rings were analysed for apoptotic cells using *in situ* Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay and supernatants were titrated for quantification of infectious virus.

6.2.5.2 Virus infection of SPF chicks

Fifty SPF chicks were allocated to 2 groups of 25 birds. The chicks in the infected group were inoculated oculonasally with $10^{5.0}$ CD_{50}/bird of the virus and those in the control group were inoculated similarly with TOC medium (Appendix I) to serve as uninfected controls. At 1, 3, 7, 9 and 14 days post infection (dpi) five chicks from each group were sacrificed and tissue samples of trachea and kidney were collected. A portion of tissue samples were stored in RNA*Later* (Qiagen, Crawley, UK) and kept at -20°C until processing for RT-PCR. The remaining portion was fixed in 10% formalin for future histopathological examination. Blood samples were

collected from 5 randomly selected chicks at 1, 7 and 14 dpi from both groups to monitor the antibody response. The clinical signs were observed daily throughout the experimental period.

6.2.6 Measurement of CEK cells metabolic activity by MTT assay

CEK cells were analysed for metabolic activity at 24 and 48 hpi using CellTiter 96, a non-radioactive cell proliferation assay, according to the manufacturer's instructions as described in Chapter 2.8.i.

6.2.7 Analysis of apoptosis by flow cytometry

CEK cells were used for analysis of apoptosis induction by staining with Annexin V-FITC and PI using commercial V-FITC apoptosis kit, according to the manufacturer's instructions (Chapter 2.8.ii).

6.2.8 Detection of apoptotic cells in TOC

Apoptotic cells were detected and quantified by the TUNEL assay using the peroxidase (POD*) in situ* cell death detection kit according to the manufacturer's instructions (Chapter 2.9). A total of five sections were analysed for each time point. For each sample, the total number of positive cells/400× microscopic field was calculated.

6.2.9 Quantification of infectious virus

Cell culture and TOC supernatants were used to titrate infectious virus in TOC expressed in CD_{50}/ml as discussed in Chapter 2.3.iii (Cook, et al., 1976b).

6.3.10 Histopathological lesions

For histopathological examination, the upper part of the trachea and anterior kidney tissues were fixed in 10% buffered formalin, embedded in paraffin and 7 µm sections were cut for hematoxylin and eosin (H&E) staining. After staining, the

sections were scored individually according to the criteria described in Chapter 2.6.ii (Chen & Itakura, 1996).

6.2.11 Quantification of the viral RNA

RNA was extracted from trachea and kidney samples using the RNeasy Plus Mini Kit following the manufacturer's instructions (Chapter 2.4.i). For quantification of viral RNA quantitative reverse transcription PCR (qRT-PCR) was carried out as described in Chapter 2.4.v.

6.2.12 Total RNA isolation and cDNA synthesis

At each time point, total RNA was extracted from trachea and kidney samples using the RNeasy Plus Mini Kit following the manufacturer's instructions (Chapter 2.4.i). RNA concentration was quantified by NanoDrop® ND-1000. The cDNA was generated from 1 µg of RNA using the Superscript III First-strand cDNA synthesis system with random primers as per the manufacturer's recommendations as described in the Chapter 2.4.iii.

6.2.13 Host gene expression analysis

QRT-PCR of cDNA samples in triplicate was performed using LightCycler 480 SYBR Green I Master mix and primers as described in the Chapter 2.4.iv. QRT-PCR data was normalized using a relative standard curve method to 18S ribosomal RNA (18S rRNA) expression (Kuchipudi, Tellabati, et al., 2012) and the data were presented as fold difference in gene expression of virus *versus* mock infected samples.

6.2.14 ELISA

Serum samples collected from all the groups at 1, 7 and 14 dpi were tested using commercial ELISA kits (IDEXX) according to the manufacturer's instructions (Chapter 2.10.i).

6.2.15 Statistical analysis

All the parametric data were analysed using the one-way analysis of variance (ANOVA), followed by the *post-hoc* least significant difference (LSD) multiple comparison test. Differences between groups were considered significant at $P<0.05$. For only the trachea and kidney lesion score and viral load the significant differences between the groups were analysed by Kruskal-Walis test followed by Dunn's mean test. Differences between groups were considered significant at $P<0.05$.

6.3 Results

6.3.1 *In vitro* study

6.3.1.1 IBV 793B infections resulted in significant increase in apoptosis of CEK cells and TOCs

CEK cells and TOCs were infected with 793B or were mock infected. CEK cell metabolic activity and percent of apoptotic cells was evaluated at 24 and 48 hpi by MTT assay and Annexin V binding assay, respectively. A significant (*P<0.05*) reduction in the cell metabolic activity was found in IBV infected CEK cells compared with mock infected cells both at 24 and 48 hpi (Figure 6. 1 A). A significantly (*P<0.05*) higher apoptosis in IBV infected CEK cells was also found at 24 and 48 hpi (Figure 6.1 B). Total apoptotic cells in IBV or mock infected TOCs were evaluated by TUNEL assay. We found that IBV infection resulted in significant increase in total apoptotic cells in TOCs compared with mock infected controls at 24 and 48 hpi (Figure 6.1 C). Notably, infection of CEK cells with 793B resulted in significantly (*P<0.05*) greater cell death as evidenced by reduced metabolic activity (Figure 6.1 A) and increased apoptosis (Figure 6.1 B) compared with mock-infected at 24 and 48 hpi. In contrast, lower infectious virus production was observed from 793B infected CEK cells and TOCs at 48 hpi compared at 24 hpi (Figure 6.1 D).

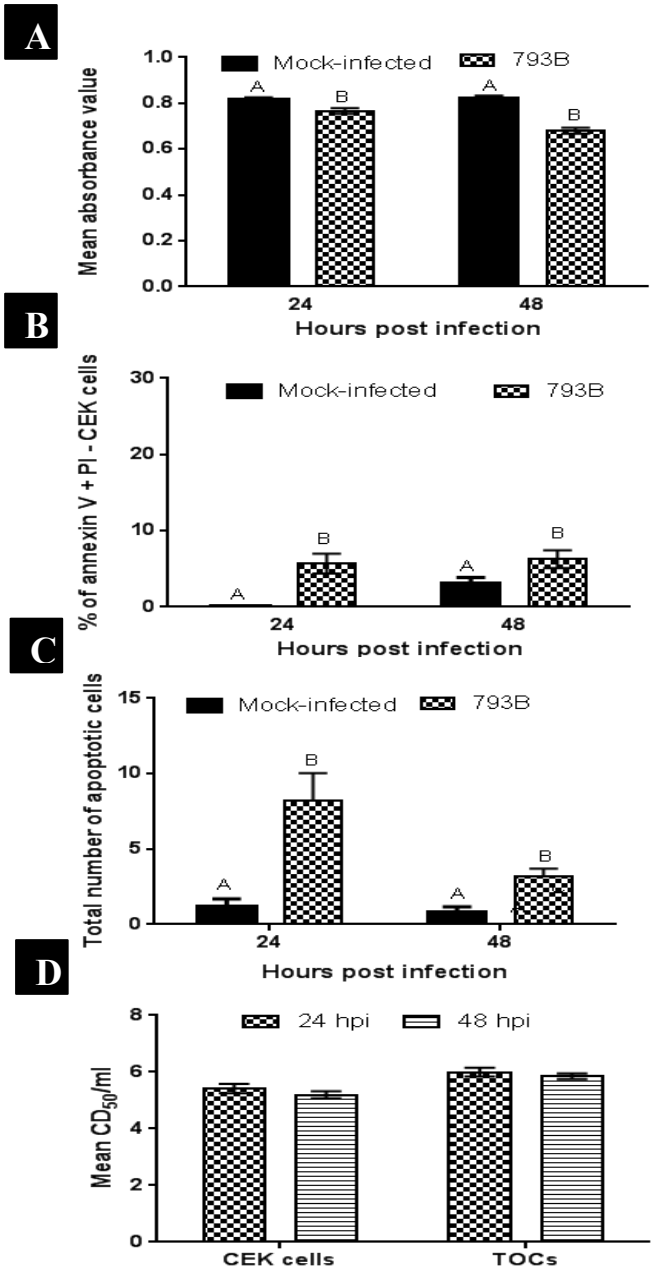


Figure 6.1. IBV 793B infections resulted in significant increase in apoptosis of chicken embryo kidney (CEK) cells and chicken embryo tracheal organ cultures (TOCs). At 24 and 48 hpi cell metabolic activity of CEK cells was evaluated by (A) MTT assay and percent of apoptosis by (B) Annexin V binding, respectively. Total apoptotic cells in TOCs were evaluated by (C) TUNEL assay. Virus production in the (D) CEK cells and TOC supernatants were determined in chicken embryo TOCs at designated time points after infection. Data represents mean of triplicate wells with error bar showing standard error. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

6.3.1.2 Host gene expression in CEK cells

CEK cells were infected with 793B or mock infected at a MOI of 1.0 and mRNA expression of INF-α, INF-β, TLR3 and MDA5 was analysed at 9 and 24 hpi as illustrated in Figure 6.2 A & B, respectively. No significant (*P<0.05*) change at 9 and 24 hpi in the levels of IFN-α (Figure 5A) expression was noticed in IBV infected CEK cells compared with mock infected cells. Notably, IFN-β expression showed no significant change at 9 hpi (Figure 6.2 A) but a significant (*P<0.05*) down-regulation at 24 hpi (Figure 6.2 B) in IBV infected CEK cells compared with mock infected cells. It was observed that expression of TLR3 and MDA5 was significantly up-regulated at 9 hpi (Figure 6.2 A) but down-regulated at 24 hpi (Figure 6.2 B) in IBV infected CEK cells compared with mock infected cells (*P<0.05*).

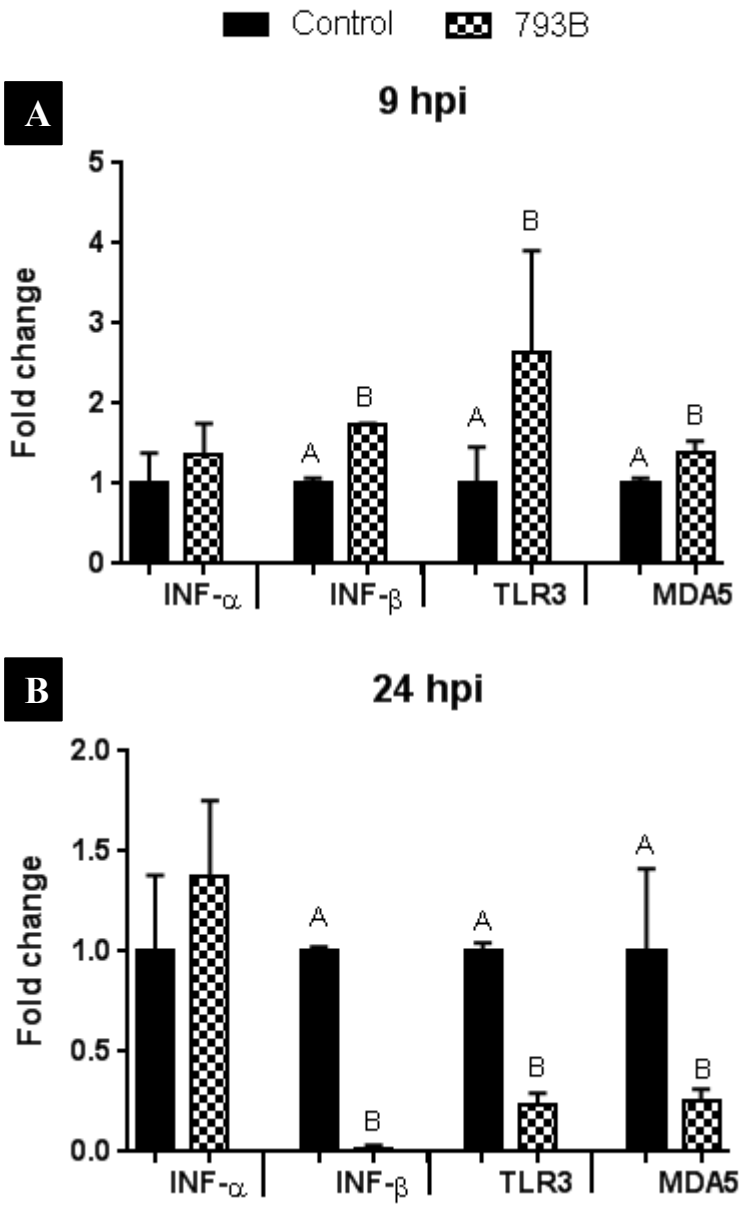


Figure 6.2. Relative mRNA expression of INF-α, INF-β, TLR3 and MDA5 in CEK cells infected with IBV strains 793B at MOI 1.0 or mock-infected and gene expression was analysed at (A) 9 and (B) 24 hours post infection (hpi). Relative mRNA expression was determined by real-time PCR normalised to 18S rRNA. Graphed values the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mocked-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

6.3.1.3 Host gene expression in TOCs

Transcriptional regulation of Type I IFN, TLR3 and MDA5 in TOCs infected with IBV strain 793B or mock infected was analysed at 9 and 24 hpi (Figure 6.3). INF-α expression was not significantly changed in TOCs at 9 hpi (Figure 6.3 A) and was down-regulated at 24 hpi in IBV infected TOCs compared with mock infected controls. There was a significant (*P<0.05*) up-regulation in the expression levels of IFN-β and TLR3 at 9 and 24 hpi (Figure 6.3) in 793B infected TOCs compared to controls. No significant change in expression level of MDA5 was observed at 9 hpi (Figure 6.3 A), but the expression was significantly (*P<0.05*) up-regulated at 24 hpi (Figure 6.3 B) in IBV infected CEK cells compared with mock infected cells.

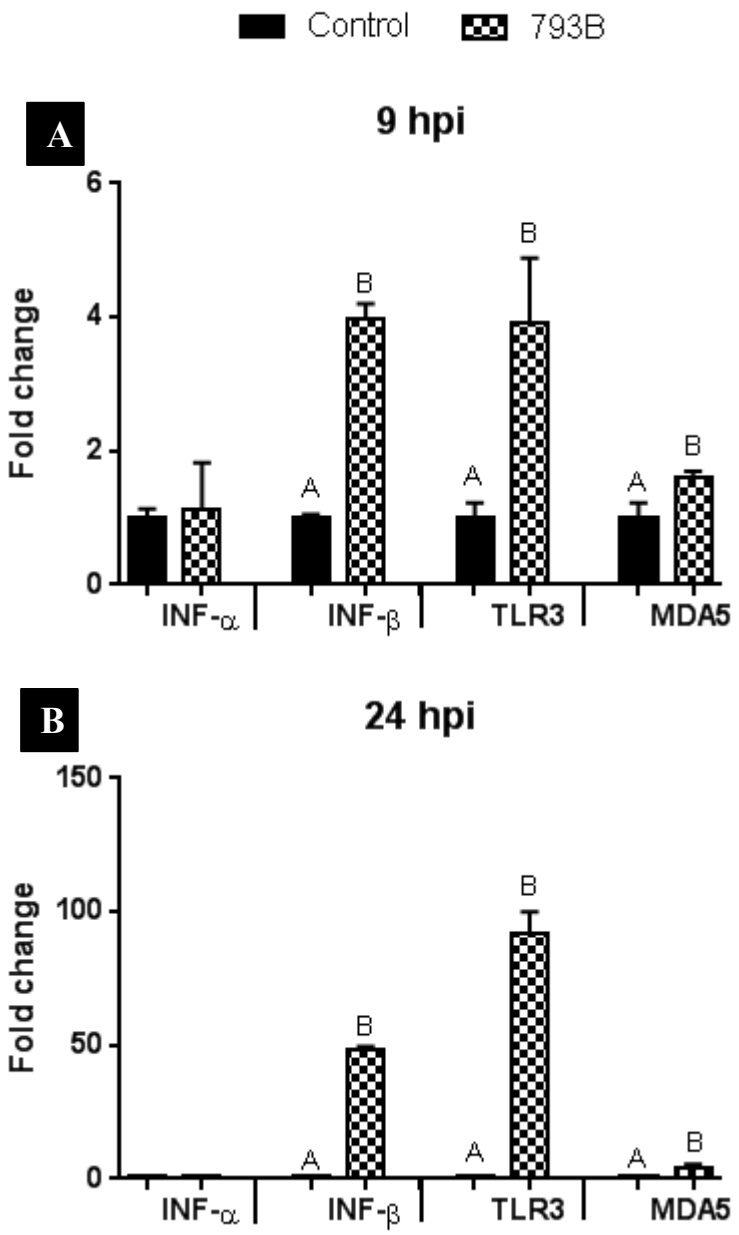


Figure 6.3. Relative mRNA expression of INF-α, INF-β, TLR3 and MDA5 TOCs infected with IBV strains 793B at a dose of 4 $\log_{10}$ CD_{50} per individual ring or mock-infected and gene expression was analysed at (A) 9 and (B) 24 hours post infection (hpi). Relative mRNA expression was determined by real-time PCR normalised to 18S rRNA. Graphed values are the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mocked-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

6.3.2 *In vivo* study

6.4.2.1 Clinical Signs

No clinical signs or mortalities were observed in the control groups during the experiment. A few chicks of the infected group showed very mild clinical signs including sneezing, coughing, head shaking and eye scratching at 2 dpi and recovered by 5 dpi.

6.3.2.2 Histopathology

Normal tracheal epithelia, with healthy cilia and goblet cells, were observed in the uninfected control birds (Figure 6.4 A). Histopathological lesions in tracheas were induced by 793B at 1 dpi. However, these lesions become more severe and peaked by 3 dpi with mean scores of 1.72 (Table 6.1). The most consistent lesions occurred mainly in the form of loss of cilia, decreased goblet cells, infiltration of lymphocytes, plasma cells and heterophils (Figure 6.4 B).

The kidneys of the chicks from uninfected control group showed no obvious lesions (Figure 6.4 C). In the 793B infected group, the main histopathological kidney lesions consisted of interstitial lymphoid infiltration with mild lymphoid nodules (Figure 6.4 D) and these changes became more severe and peaked by 9 dpi with mean scores of 1.3 (Table 6.2)

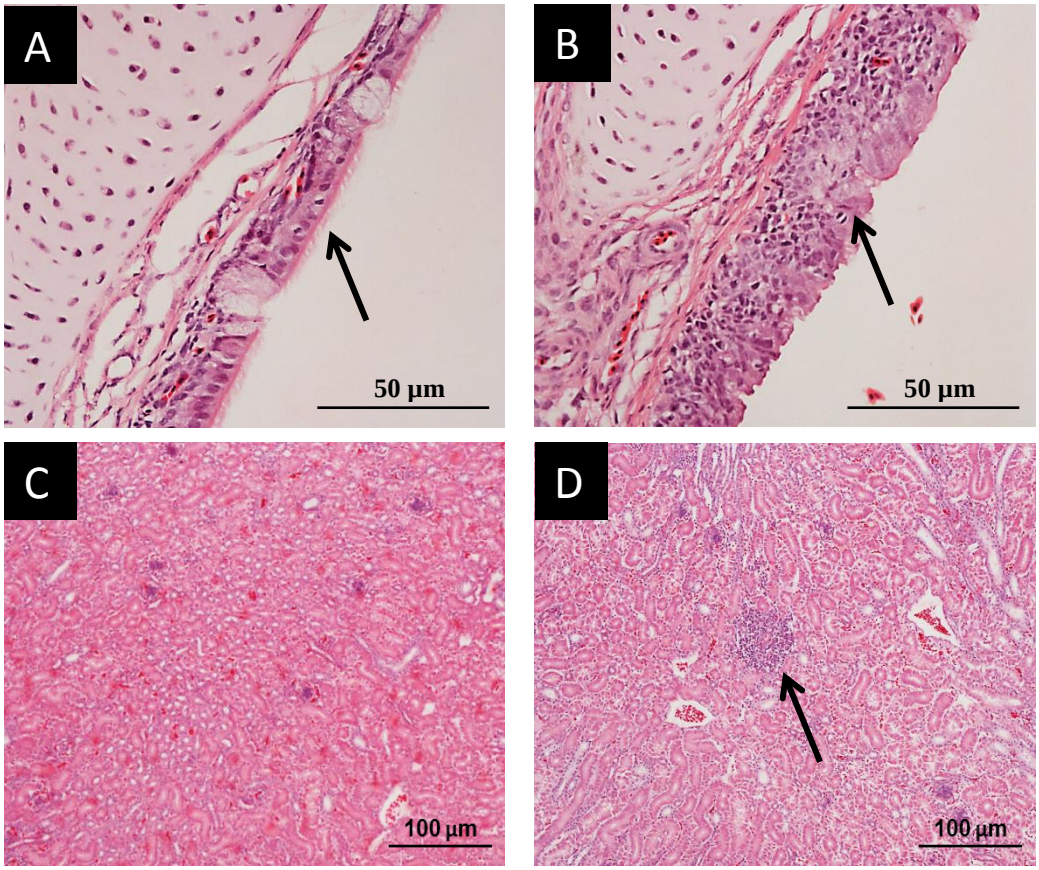


Figure 6.4. Histopathological findings in trachea (at 3 dpi) kidney (at 9 dpi) samples, using Haematoxylin and Eosin (H&E) stains, (A) Uninfected control group trachea (B) 793B infected group trachea showing mild epithelial deciliation, with moderate lymphocyte and heterophil infiltration (C) Uninfected control group kidney (D) 793B infected group kidney showing mild leucocyte interstitial infiltration.

Table 6.1. Histopathology of the trachea in SPF chicks infected with IBV 793B strain

Days post infection	Bird No.	Epithelial deciliation	Epithelial degeneration	Decrease mucous cells	Heterophil infiltration	Epithelial hyperplasia	Lymphoid infiltration	Total score	Mean score±SE
1	1	0	0.5	0	0	0	0	0.5	0.7±0.33
	2	0	0	0	0	0	0	0	
	3	0	0	0	0	0	0	0	
	4	0.5	1	0	0	0	0	1.5	
	5	0.5	0	1	0	0	0	1.5	
3	1	0	0	0	0	0	0	0	3.5±1.72
	2	0	0	0	0	0	0	0	
	3	2	1	2	1	1	1.5	8.5	
	4	1	1.5	0	0	0	0	2.5	
	5	1.5	1.5	0	1	1	1.5	6.5	
7	1	2.5	1	3	0.5	1	2	10	8.3±0.71
	2	3	1	2	0.5	0	1	7.5	
	3	2	1	2.5	0	1	2	8.5	
	4	2.5	1	1	0.5	0	1	6	
	5	2	1	3	0.5	0.5	2.5	9.5	
9	1	0	1	0	0	1	1	3	4.1±1.14
	2	1	0.5	3	0	1	1	6.5	
	3	1.5	0.5	3	0	1	1	7	
	4	1	0.5	0.5	0	0.5	0.5	3	
	5	0.5	0	0	0	0	0.5	1	
14	1	0	0.5	0.5	0	0.5	1	2.5	1.5±0.47
	2	0	0	0	0	1	1.5	2.5	
	3	0	0	0	0	0	0	0	
	4	0	0	0	0	0.5	1	1.5	
	5	0	0	0	0	0.5	0.5	1	

Data are expressed as the mean histopathological lesion score ± SEM (n = 5). Tracheal lesions scores as follows: no change (0), mild (1), moderate (2) or severe (3).

Table 6.2. Histopathology of the kidney in SPF chicks infected with IBV 793B strain

Days post infection	Bird No.	Epithelial deciliation	Ducto-tubular dilation	Heterophil infiltration	Lymphoid infiltration	Epithelial degeneration	Epithelial hyperplasia	Lymphoid nodules	Fibroblastic proliferation	Total score	Mean score±SE
1	**1**	0	0	0	0	0	0	0	0	0	0.2±0.12
	2	0	0	0	0	0	0	0.5	0	0.5	
	3	0	0	0	0	0	0	0.5	0	0.5	
	4	0	0	0	0	0	0	0	0	0	
	5	0	0	0	0	0	0	0	0	0	
3	**1**	0.5	0	0	0	0	0	0	0	0.5	0.4±0.10
	2	0	0	0	0	0	0	0.5	0	0.5	
	3	0	0	0	0	0	0	0.5	0	0.5	
	4	0	0	0	0	0	0	0.5	0	0.5	
	5	0	0	0	0	0	0	0	0	0	
7	**1**	0	0	0	0	0	0	0	0	0	0.40±0.40
	2	0	0	0	0	0	0	0	0	0	
	3	0	0	0	1	0	0	1	0	2	
	4	0	0	0	0	0	0	0	0	0	
	5	0	0	0	0	0	0	0	0	0	
9	**1**	0.5	0	0	0.5	0	0	0	0	1	1.3±0.43
	2	0	0	0	0.5	0	0	0	0	0.5	
	3	0	0	0	0.5	0.5	0	0	0	1	
	4	0.5	0	0	0.5	0	0	0	0	1	
	5	0.5	0.5	0	2	0	0	0	0	3	
14	**1**	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
	2	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
	3	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
	4	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	
	5	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	*ND*	

ND: not done as sample lost during processing; Data are expressed as the mean histopathological lesion score ± SEM (n = 5). Kidney lesions scores as follows: no change (0), mild (1), moderate (2) or severe (3).

6.3.2.3 Viral RNA quantification in trachea and kidney

Viral RNA load in the tracheas of birds of uninfected control group on all days post infection were below the detection limit (Figure 6.5). The viral RNA load in trachea the infected group was detected at 3 dpi and the peak was observed at 7 dpi. In kidneys of infected chicks viral RNA load was first detected at 7 dpi and peak load was observed at 9 dpi.

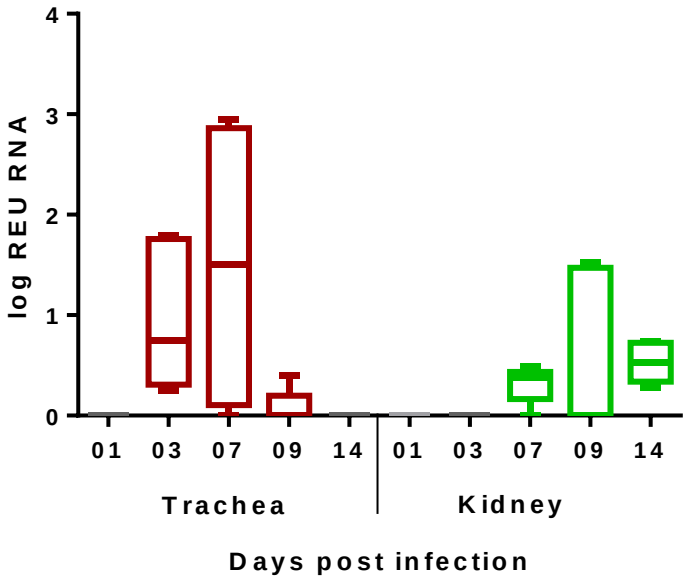


Figure 6.5 Quantification of viral RNA, expressed as a log relative equivalent units (REU) of RNA in Trachea and Kidney samples of chicken. Birds (n=5/group) were infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and analysed for quantification of viral RNA at 01, 03, 07, 09, and 14 days post infection (dpi). Data represents the mean with error bars as standard error. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test indicated with different letters (*P<0.05).*

6.3.2.4 Relative IFN-α and IFN-β mRNA expression

The mRNA expression data for IFN-α and IFN-β in the trachea are illustrated in Figure 6.6 A & B, respectively. No significant change in the IFN-α mRNA expression levels at any dpi was noticed in the infected group compared with uninfected control group (Figure 6.6 A). After peaking at 1 dpi, tracheal samples of chicks infected with 793B resulted in significantly (*P<0.05*) greater IFN-β mRNA expression at 1, 3, 7 dpi compared with uninfected control group (Figure 6.6 B). Conversely, on subsequent dpi no significant changes in level of IFN-β mRNA expression were detected in infected group compared with uninfected control group.

The mRNA expression data for IFN-α and IFN-β in the kidney is illustrated in Figure 6.7 A and B, respectively. No significant (*P<0.05*) differences in the IFN-α expression levels at any dpi was noticed in any infected group compared with uninfected control group. At 1 and 3 dpi, IFN-β mRNA expression in kidney samples of chicks infected with 793B was up-regulated (*P<0.05*) compared with uninfected control group. No significant changes in level of IFN-β expression was observed in any infected group at the other time points (7, 9 or 14 dpi).

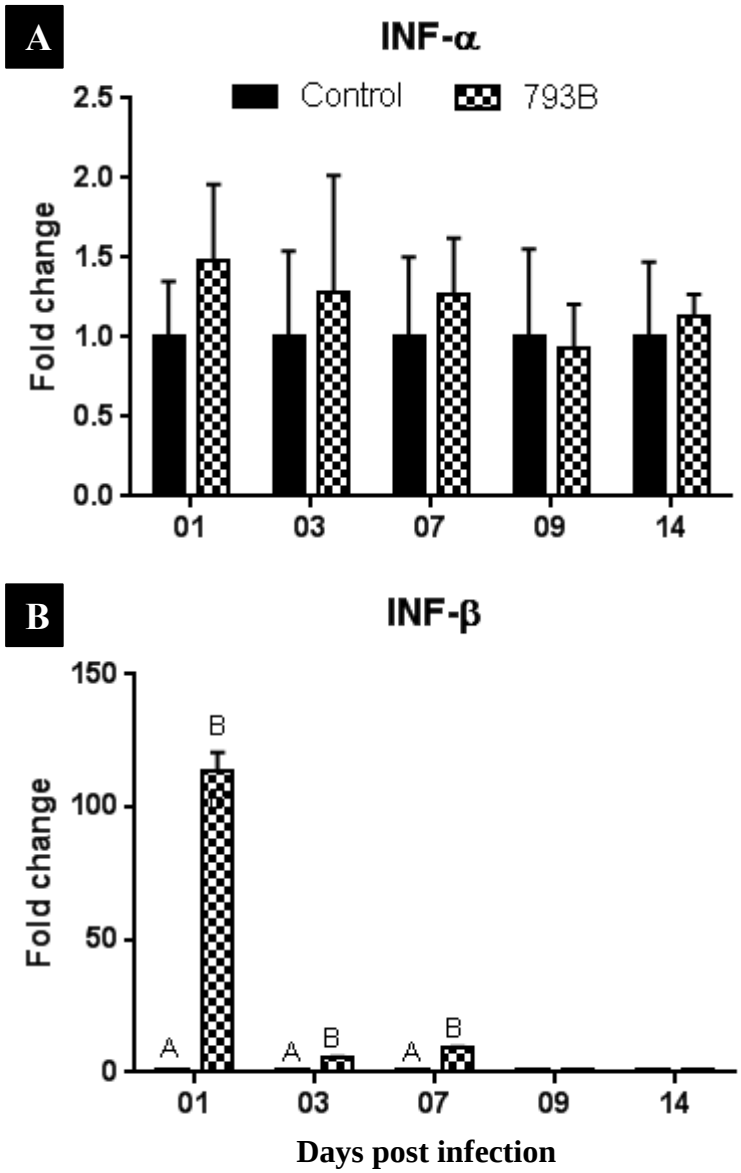


Figure 6.6. Relative Type I interferon (IFN) (A) IFN-α and (B) IFN-β mRNA expression in trachea of chickens (n=5/group) infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

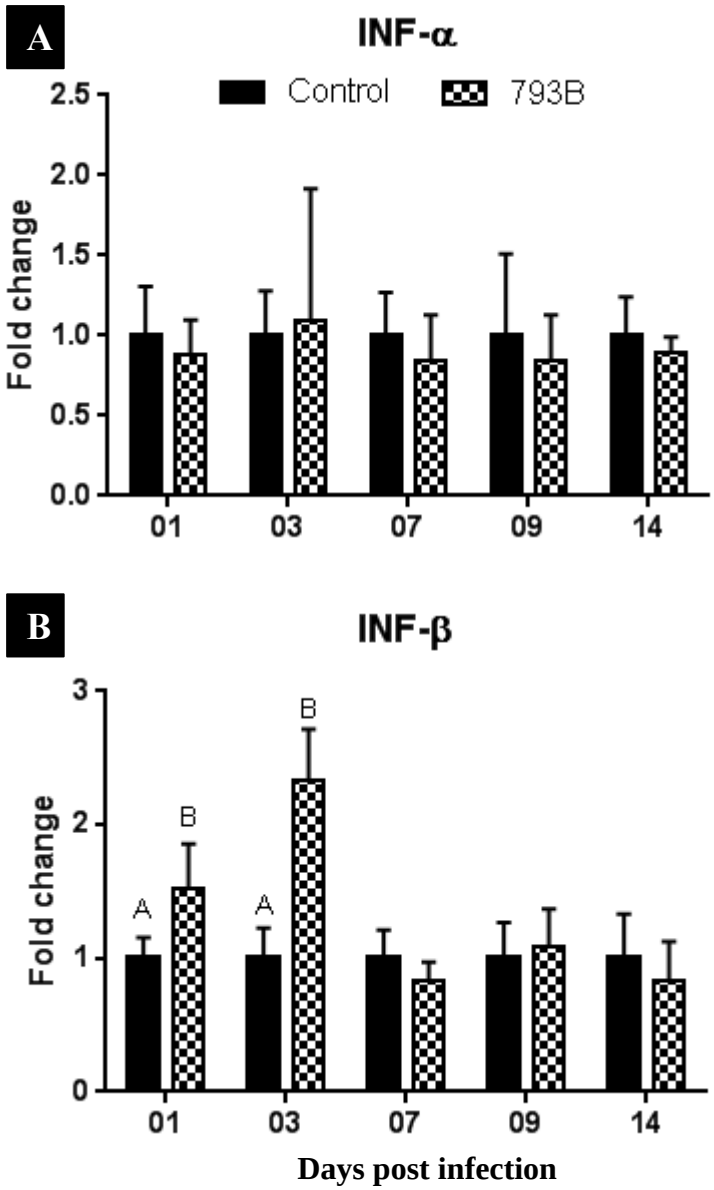


Figure 6.7. Relative Type I interferon (IFN) (A) IFN-α and (B) IFN-β mRNA expression in kidney of chickens (n=5/group) infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

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

The mRNA expression of TLR3 and MDA5 in the tracheal samples from chicks infected with IBV strain 793B or the uninfected control group are illustrated in Figure 6.8 A & B, respectively. It was observed that expression of TLR3 (Figure 6.8 A) in the infected group was significantly (*P<0.05*) up-regulated at 1 and 3 dpi compared to the uninfected control group. No significant (*P<0.05*) changes in TLR3 mRNA expression levels were detected in the infected group compared with the uninfected control group on subsequent dpi. Similarly, 793B infection resulted in significantly (*P<0.05*) greater MDA5 expression in the trachea at 1 dpi compared with the uninfected control group (Figure 5.9 B). No significant changes in level of MDA5 expression was observed in the infected group at the other time points (3, 7, 9 or 14 dpi).

The mRNA expression data for TLR3 and MDA5 in the kidney is illustrated in Figures 6.9 A and B, respectively. We found that 793B infection resulted in significantly (*P<0.05*) higher mRNA expression of TLR3 (Figure 6.9 A) compared with the uninfected control group only at 3 dpi. Notably, no significant changes in level of TLR3 mRNA expression were observed in any infected group at the other time points (1, 7, 9 or 14 dpi) compared with the uninfected control group. At 3 and 7 dpi, MDA5 mRNA expression (Figure 6.9 B) in kidney samples of chicks infected with 793B was up-regulated and was significantly (*P<0.05*) higher compared with the uninfected control group.

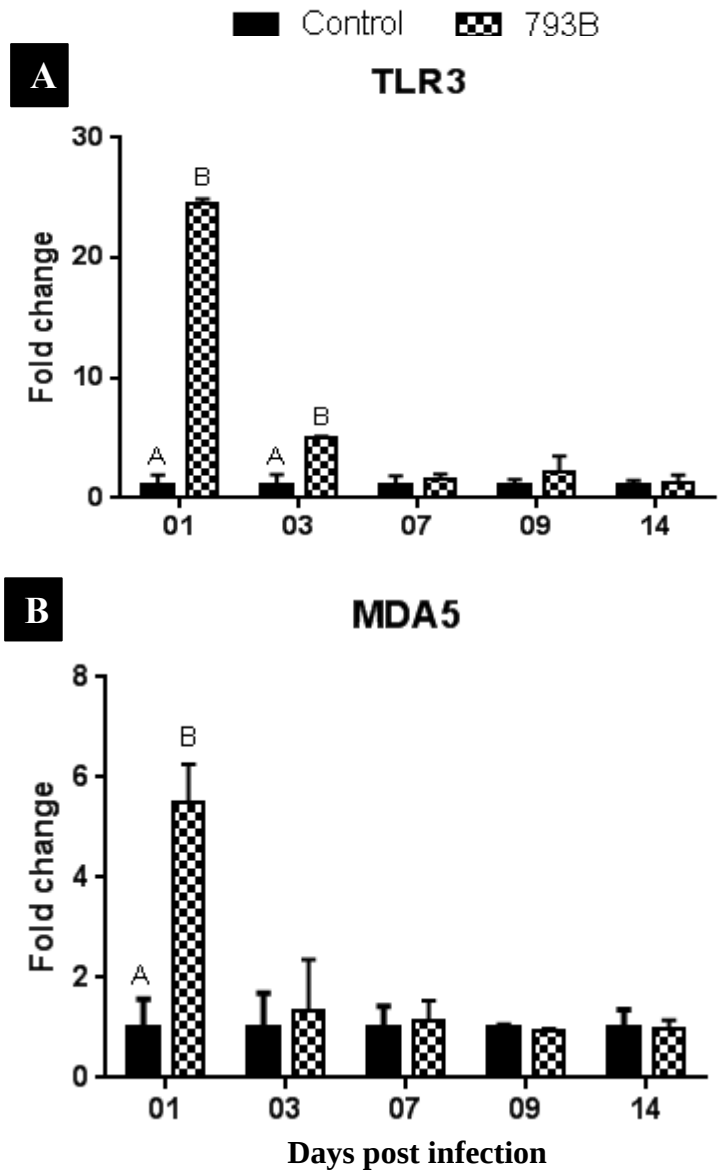


Figure 6.8. Transcriptional regulation of innate viral sensing molecules (A) TLR3 and (B) MDA5 in trachea of chicken (n=5/group) infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

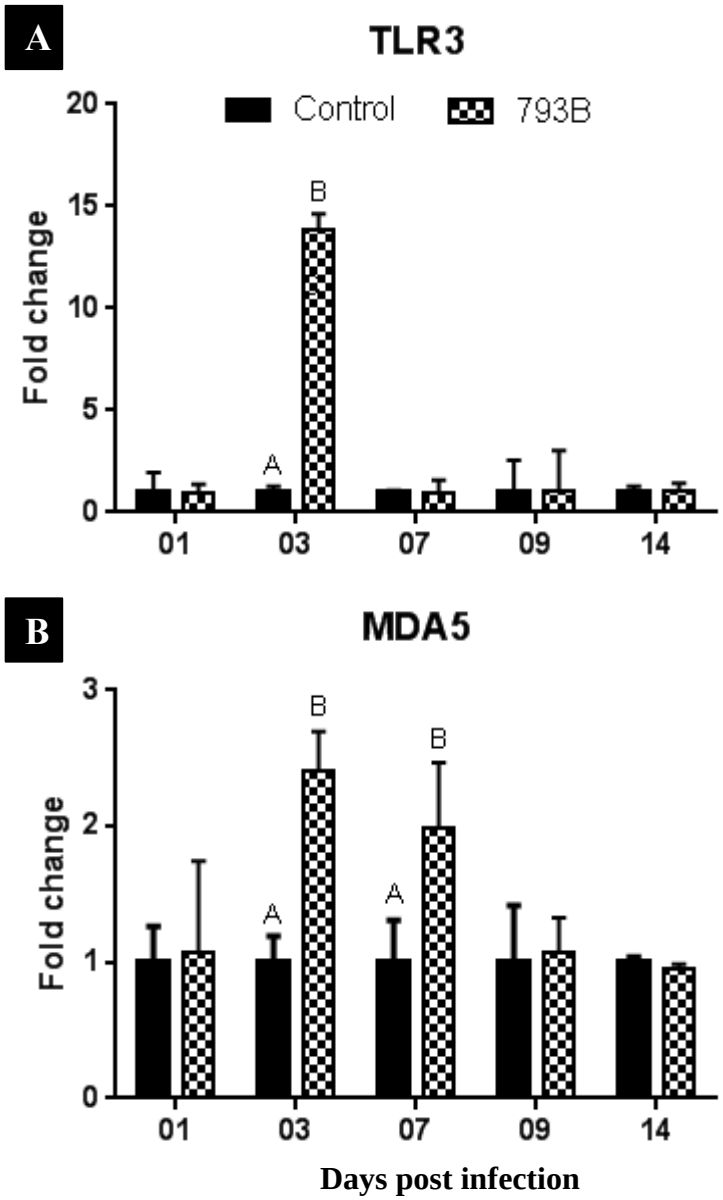


Figure 6.9. Transcriptional regulation of innate viral sensing molecules (A) TLR3 and (B) MDA5 in kidney of chicken (n=5/group) infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean of three biological replicates with error bars as standard error and are expressed as fold change relative to the mock-infected group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

6.3.2.6 Transcription profile of proinflammatory cytokines in the trachea and kidney

The changes in levels of 3 pro-inflammatory cytokines in trachea are illustrated in Figure 6.10. The results indicate that, the level of IL-1β mRNA expression peaked at 7 dpi, with mRNA expression being significantly *(P<0.05)* higher at 3 and 7 dpi in chicks infected with 793B compared with uninfected control. Figure 6.10 B and C shows the expression of IL-6 and LITAF, respectively wherein, at 7 dpi, a peak increases in the expression was noticed in chicks infected with 793B. However, mRNA expression was significantly *(P<0.05)* higher at 3 and 7 dpi in chicks infected with 793B compared with uninfected control.

Conversely, in the kidney samples, IL-1β mRNA expression level expression peaked at 9 dpi in all infected group and was significantly *(P<0.05)* up-regulated in 793B infected group compared to uninfected control group (Figure 6.11 A). At 7 and 9 dpi, IL-1β mRNA expression in infected group was significantly (*P<0.05*) up-regulated compared with uninfected control group. The mRNA expression of IL-6 and LITAF in kidneys from the infected group peaked at 14 dpi (Figure 6.11 B & C) and was significantly (*P<0.05*) up-regulated compared with uninfected control group but. However, at 7 and 9 dpi, IL-6 and LITAF mRNA expression level was significantly *(P<0.05)* up-regulated in 793B infected group compared to uninfected control group.

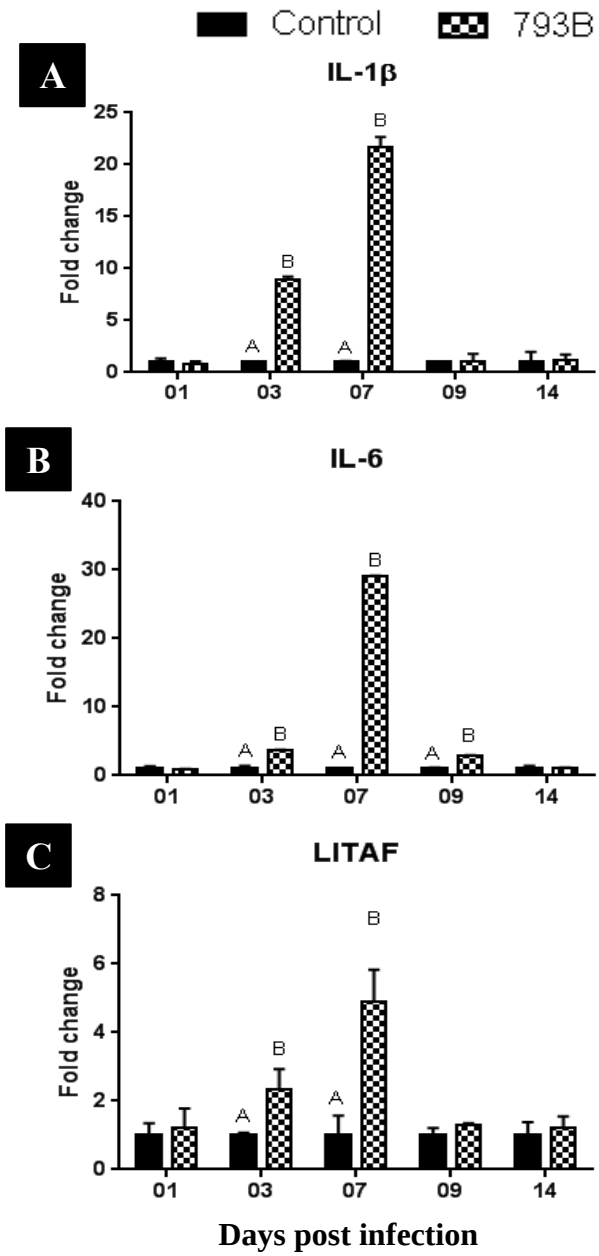


Figure 6.10. Transcription profile of proinflammatory cytokines (A) IL-1β, (B) IL-6 and (C) LITAF in trachea of chicken (n=5/group) infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

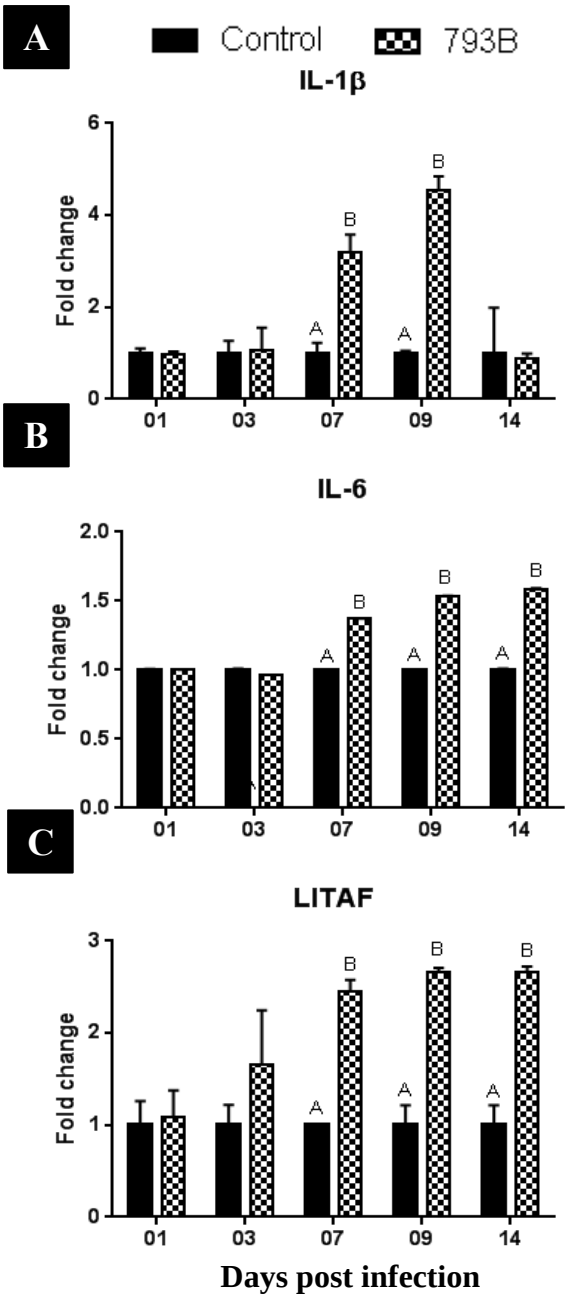


Figure 6.11. Transcription profile of proinflammatory cytokines (A) IL-1β, (B) IL-6 and (C) LITAF in kidney of chicken (n=5/group) infected with IBV strain 793B at a dose of $10^{5.0}$ CD_{50}/bird and gene expression was analysed at 01, 03, 07, 09, and 14 days post infection (dpi). Relative mRNA expression was determined by quantitative reverse transcription PCR (qRT-PCR) using SYBR Green method and the data were normalised to 18S rRNA expression. Data represents the mean with error bars as standard error and are expressed as fold change relative to the uninfected controls group. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test indicated with different letters (*P<0.05)*.

6.3.2.7 ELISA

Serum samples from both the groups collected prior to infections were free of the IBV antibodies (Figure 6.12). At 7 and 14 dpi, all chicks inoculated with 793B showed a positive IBV antibody response with significant (*P<0.05*) difference compared with uninfected control group.

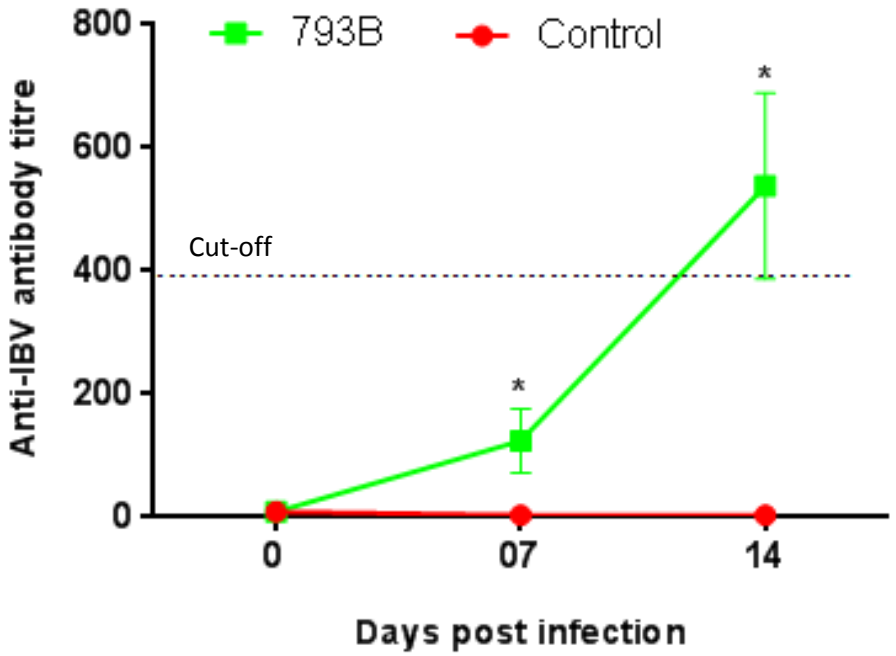


Figure 6.12. Infectious bronchitis virus (IBV) antibody titres of the different groups (n=5/group) infected with IBV strains 793B or uninfected control at a dose of $10^{5.0}$ CD_{50}/bird. Data is expressed as mean values ± SEM. Significant differences between the groups were detected by one-way ANOVA, followed by the *post-hoc* LSD multiple comparison test (*P<0.05)*. Asterisks indicate that values between the groups were significantly different *(P<0.05)* at those time point. Cut-off point titre = 396.

6.4 Discussion

Infectious bronchitis virus (IBV) 793B occurs frequently in vaccinated and non-vaccinated flocks and causes severe economic losses. In the present study, immunopathogenesis of 793B serotype was comprehensively analysed after *in vitro* and *in vivo* infections.

To assess the effect of *in vitro* infection, we infected CEK cells and TOCs with 793B or mock infected. Overall a significant higher death rate of CEK cells was observed in the infected group compared with mock-infected. We found that IBV infection resulted in increased apoptosis in CEK cells as well as in TOCs. As discussed in Chapter 3 and 4, that IBVs are known to induce apoptosis in infected cells and B-cell lymphoma 2 (Bcl-2) family proteins modulate IBV-induced apoptosis (Li, et al., 2007; Liu, et al., 2001; Zhong, Liao, et al., 2012; Zhong, Tan, et al., 2012). Our findings suggest a correlation between apoptosis in CEK cells and TOCs and the ability of 793B to cause kidney or respiratory lesions respectively in chicken. The potential for apoptosis to cause such tissue damage has also been recognized for the Coronavirus infections (SARS-CoV) (Tan, et al., 2004) which was discussed in Chapter 3.

Another objective of *in vitro* study was to analyse the early innate mechanism involved in 793B infection of CEK cells and TOCs. Infection of 793B resulted in significantly higher up-regulation of innate immune sensing genes TLR3 and MDA5 along with higher IFN-β mRNA levels in CEK cells and TOCs at 9 hpi than mock-infected group. Notably, expression of all the innate immune genes tested, was down-regulated at 24h after IBV infection in CEK cells. As discussed in Chapter 3, this could be a consequence of host shut-off induced by IBV virus replication

(Kamitani, et al., 2006; Tohya, et al., 2009). In addition, coronavirus N protein has been shown to interfere with induction of Type I IFN, which in turn can also inhibit the production of various pro-inflammatory cytokines and chemokines via global translational shutdown (Zhong, Tan, et al., 2012). However, TLR3, MDA5 and IFN-β expression was up-regulated in TOCs at 24 hpi. The difference between the expression profile of the innate immune genes at 24 hpi in CEK cells and TOCs could be due to difference in the dynamics of virus replication.

Several *in vitro* and *ex vivo* studies have suggested a key role of TLR3 in viral detection (Alexopoulou, et al., 2001; Bowie & Haga, 2005; Finberg & Kurt-Jones, 2004; Tabeta, et al., 2004). TLR3 has been reported (discussed in Chapter 3 and 4) to contribute to detrimental effects caused by West Nile virus in mice (Wang, et al., 2004) and influenza virus infection (Le Goffic, et al., 2006). A recent study showed that MDA5, but not TLR3, is involved in the sensing of IBV (Kint, et al., 2015). However, in the *in vivo* pathogenicity part of present study (discussed later), we observed that mRNA levels of TLR3 and MDA5 were comparable with the pattern of histopathological lesion scores in trachea and kidney produced by 793B. This could be explained by the hypothesis that MDA5 activation is a protective antiviral host response against the virus whereas TLR3 contributes to the detrimental effects of the viral infection. However, further studies should be performed to confirm this hypothesis. In addition, significantly lower infectious virus production was observed from 793B infected CEK cells and TOCs supernatant at 9 and 24 hpi. This could be consequential with the increased antiviral state induced by IFN-β and/or increased apoptosis as discussed in Chapter 3 and 4.

In this study we also investigated the immunopathogenesis in SPF chicks infected with 793B. Our findings showed that chicks responded to 793B infection as chicks displayed very mild clinical signs but no mortalities. In agreement with this, it has been reported that the birds inoculated with 793B serotype unlikely to cause mortality or severe clinical signs in infected chicks in uncomplicated cases (Benyeda, et al., 2010; Bijanzad, et al., 2013; Otsuki, et al., 1990). We also found a positive IBV antibody response with significant difference with control at 7 and 14 dpi in chicks infected with 793B which shows the establishment of infection.

We noted that infection of 793B resulted in obvious histopathological changes in the trachea and kidney similar to those reported in previous experiments (Bijanzad, et al., 2013; Chen *et al.*, 1996; Chousalkar, et al., 2007; Kotani, et al., 2000). These pathological changes in the trachea peaked by 7 dpi in the trachea and by 9dpi in kidney. A similar trend was found for viral load in trachea and kidney. Interestingly, these peak values of histopathological changes and viral load were found to be associated with peaks of expression of inflammatory cytokines, such as IL-6 and IL-1β, and higher LITAF mRNA expression values. This suggests a role of both these cytokines and virus load in the development of pathological lesions. Similar findings were reported after experimental infection with IBV QX (Jang, et al., 2013) and other strains (discussed in Chapter 5). An association between IL-6 and IL-1β with high virus loads and the development of tracheal lesions has also been observed following IBV-M41 infection (Okino, et al., 2014)

The role of TLR3 in viral immunology has been discussed in Chapter 3 (Akira, 2001; Le Goffic, et al., 2007). It was observed that 793B infection resulted in significantly higher up-regulation of TLR3 levels in trachea at 1 dpi and in kidney at 3 dpi.

Parallel with our findings, previous studies have also reported a significant increase of TLR3 mRNA expression in trachea of IBV infected chicks when compared to the uninfected controls (Kameka, et al., 2014; Wang, et al., 2006). In contrast, in the most recent study, no significant changes was observed in TLR3 expression in kidney following infection with attenuated CK/CH/LHLJ/04V strain, which could be due to loss of virulence (Xu *et al.*, 2015). In our study, MDA5 mRNA expression values peaked in 793B infected trachea and kidney at 1 and 3 dpi, respectively. MDA5 mRNA expression levels were reported to be significantly increased in chicken kidney tissue after nephropathogenic IBV infection suggesting a role of chicken MDA5 in IBV infection (Cong, et al., 2013).

In addition, it has been reported that *in vitro* virulent IBV infection leads to a significant induction of IFN-β transcription through an MDA5- dependent activation of the IFN response (Kint, et al., 2015). Concurrent with TLR3 and MDA5 activation, we found a significant up-regulation of IFNβ but not IFNα mRNA expression at 1 dpi in trachea and 3 dpi in kidney after IBV infections. Many previous studies claims that the activation of the TLR3 pathway works towards an up-regulation of INF-β production in chicks (Kameka, et al., 2014; Karpala, et al., 2008; Parvizi, et al., 2012). Altogether, in this study we found that mRNA levels of TLR3 and MDA5 showed the same pattern as that of histopathological lesion scores in trachea and kidney produced by 793B.

This study represents some of the underlying mechanisms of induction of host responses by 793B in chicks as well as *in vitro*. We are reporting that *in vitro* higher apoptosis together with elevated levels of TLR3, MDA5 and IFN-β expression correlates with *in vivo* pathogenicity of 793B in tracheas and kidneys. Additionally,

in vivo results provided further evidence that the higher upregulation of expression of proinflammatory cytokines (such as IL-6 and IL-1β) and LITAF induced by 793B in trachea and kidney of SPF chicks may be ascribed to histopathological damage caused by the virus in those respective organs.

Chapter 7: Mucosal, cellular and humoral immune responses induced by different live infectious bronchitis virus vaccination regimes and the protection conferred against infectious bronchitis virus Q1 strain

The data from this Chapter have been published (Appendix III) in peer-reviewed journal as below:

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7.1 Introduction

The prevention of infectious bronchitis (IB) in chickens is achieved through the use of live and inactivated vaccines, which provide protection against virulent field IB viruses in the event of an exposure. Despite these preventative measures, outbreaks of IB frequently occur in many poultry producing countries (Gelb, Wolff, et al., 1991; Gough, et al., 1992; Liu & Kong, 2004). This is probably due to the emergence of new variants of infectious bronchitis virus (IBV) (Gelb, Wolff, et al., 1991; Gough, et al., 1992; Jia *et al.*, 1995; Liu & Kong, 2004; Pohuang *et al.*, 2009). For the successful protection of chickens against infection, it is essential to identify the prevalent genotypes in the region and to determine the cross-protective potential of available vaccines and optimise strategic vaccination programmes.

IB was first described in the USA during the 1930s and was identified in the UK in 1948. Thereafter, many IBV variants were isolated from Europe, significantly a variant called 793B that emerged in the 1990s (Parsons, et al., 1992). Later, IBV QX was first identified in China (Wang Y, 1998) before spreading to Europe (Worthington, et al., 2008). Another IBV genotype, Q1, genetically and serologically distinct from the classical IBVs, was also reported in China (Yu, et al., 2001), the Middle East (Ababneh *et al.*, 2012) and Europe (Toffan, et al., 2011). To contain this strain, an effective vaccination programme is needed. However, very little is known about the cross protection induced by the commercially available vaccines or vaccination regimes against this variant Q1.

An effective and long-lasting protection against IBV infection requires the activation of effector, memory cell-mediated and humoral immune responses against the virus (Cavanagh, 2007). A number of studies have reported the systemic and local

humoral immune response (HIR) to IBV vaccination (Cavanagh, 2007; Ignjatovic & Galli, 1994; Mockett & Darbyshire, 1981). In chickens, experimentally challenged with IBV, the development of a cell mediated immune response (CMI) has been correlated with effective virus clearance, reduction of clinical signs and resolution of lesions (Collisson, et al., 2000). The presence of cytotoxic CD8+ T lymphocytes (CTL) represents a good correlation for decreasing infection and corresponds with a reduction in clinical signs, as CTL activity is major histocompatibility complex restricted and these T cells mediate cytolysis (Pei, et al., 2001). It has additionally been shown that the transfer of CTLs obtained from spleen of IBV-infected chickens, was protective to naïve chicks against a subsequent IBV challenge (Collisson, et al., 2000; Seo, et al., 2000). During the course of experimental viral infection, Kotani *et al* (2000) showed that the clearance of the IBV from the tracheal mucosa occurred at an early phase of the infection and CTLs at the tracheal mucosa were proposed to be involved in this clearance (Kotani, et al., 2000). To date, there is no information available on the tracheal mucosal leukocytes after vaccination with live IBV vaccines. Nevertheless, Okino *et al* (2013) have quantified the relative expression of the CTLs genes in tracheal samples from vaccinated and further challenged birds. The up regulation of these genes, in the tracheal mucosa of the full-dose vaccinated birds, was significantly increased at 24 hours post infection (hpi), demonstrating the development of a memory CMI (Okino, et al., 2013). However, these researchers did not directly measure the activity of CMI, such as the cytotoxic mechanism of CTLs.

Despite all these reports, the kinetics of, and the relationship between local and systemic HIR and CMI induced by different IBV vaccination regimes, needs to be

better understood for protection against emerging IBV strains. Thus, the objective of our study was to measure the local as well as systemic HIR and CMI induced by two different IBV vaccination regimes administered to commercial broiler chicks, and to estimate the protection achieved against a recently isolated virulent Q1 strain.

7.2 Materials and methods

7.2.1 Chicks

One hundred twenty broiler chicks with IBV MDA, aged 1-day-old, were obtained from a commercial hatchery (Chapter 2.5.i). Chicks were kept in an isolation unit (University of Liverpool) throughout the experiment and reared on deep litter with water & feed provided *ad libitum* as described in Chapter 2.5.ii.

7.2.2 Challenge virus

The virulent Q1 isolate used in this study was kindly provided by Merial Animal Health. RT-PCR (as described Chapter 2.4.vi) followed by sequencing (Chapter 2.5.vii) confirmed that the allantoic fluid, from eggs used to propagate the virus, was free of Newcastle disease virus, avian influenza virus, infectious bursal disease virus, infectious laryngotracheitis virus and avian metapneumovirus. Q1 IBV was also free of bacterial or fungal contaminants. The virus was titrated in the chicken tracheal organ culture (TOC) as described in Chapter 2.3.iii and expressed in 50% (median) ciliostatsis doses (CD_{50})/ml (Cook *et al.*, 1976a).

7.2.3 Vaccine preparation

As recommended by the manufacturer (Merial Animal Health Limited, UK), the vaccines were prepared by thoroughly mixing one vial of live IBV (Massachusetts-type) H120 (Bioral H 120®) vaccine with 100 ml of sterile water (SW). For combined

vaccinations, one vial of each Bioral H 120® and live IBV (793B-type) CR88 (GALLIVAC® IB88) vaccines were mixed together in 100 ml of SW. Immediately after preparation, the vaccines and SW were kept in a cold box (at 0°C). Each chick received a total of 100 µl of the appropriate vaccine ocularly (50 µl) and nasally (50 µl) or SW. To quantify the virus, titration of live IBV vaccine for H120 and CR88 was performed by using 9-11 days of age (doa) specific pathogen free (SPF) embryonated chicken eggs (ECE) inoculated via the allantoic cavity (Chapter 2.3..iii). The ECE were examined for IBV specific lesions (curling and dwarfing) of the embryos up to 5 days post inoculation. Viral titres were calculated as described earlier (Reed & Muench, 1938) and expressed as the egg infective dose (EID_{50}/ml). The titre of the vaccine viruses used was 3.5 $\log_{10}$ EID_{50}/chick and 4.25 $\log_{10}$ EID_{50}/chick for the H120 strain and CR88 strain, respectively.

7.2.4 Experimental design

One hundred and twenty broiler chicks, aged 1-day-old, were divided into three groups (n=40 chicks/group) (Table 7.1). Chicks in group I were inoculated oculonasally with 100 µl of live H120 vaccine alone. In group II, chicks were inoculated oculonasally with 100 µl of both live H120 and CR88 vaccines simultaneously. Chicks in both groups (I and II) were again inoculated with a live CR88 vaccine at 14 doa. Group III received only 100 µl of SW oculonasally and was kept as a control. Samples (5 birds/group) of serum, tears and heparinized blood were collected at 0, 4, 7, 14, 21 and 28 doa before sacrificing the birds. The tears and serum samples were stored at -20°C, and blood samples were processed immediately for peripheral blood mononuclear lymphocytes isolation. Five chickens from each group per interval were humanely euthanized for the collection of

approximately 1 cm of the upper trachea in OCT to be snap-frozen in liquid nitrogen for immunohistochemistry (IHC). The rest of the trachea was used for tracheal washes. At 28 doa, 10 birds from each group were challenged via ocular-nasal route with Q1 ($10^{4.0}$ CD_{50}/bird) and observed daily for clinical signs. After 5 days post challenge (dpc), all 10 birds from each group were necropsied and tracheal samples were collected; a portion placed in the RNA*Later* and stored at -70°C until processing for examination of viral RNA load. The remaining portions were examined by histopathology and ciliostatsis test. The kidneys from all groups were also taken for histopathology and viral RNA load examination.

Table 7.1. Study design showing groups, vaccine and vaccination regimes. At 28 day of age, 10 chicks from each group were challenged with a virulent IBV Q1.

IBV vaccine (dosage/chick in 100 µl)	**Group/days of age**					
	I		**II**		**III**	
	0	**14**	**0**	**14**	**0**	**14**
H120 ($3.5 \log_{10} EID_{50}$)	√		√			
CR88 ($4.25 \log_{10} EID_{50}$)		√	√	√		
Sterile water (SW)					√	√

7.2.5 Sample collection for antibody detection

The potential of the vaccines to induce antibody production was assessed individually by using samples of sera, tears and tracheal washes. Tears were collected using sodium chloride as described before (Ganapathy *et al.*, 2005), immediately centrifuged at 3000 x g for 3 min before storing the supernatant at -70°C until used. To collect the tracheal washes, the trachea was clamped with two artery forceps at both the ends, and washed with 1 ml PBS using a syringe with 19 gauge needle (Raj & Jones, 1996c). The collected samples were centrifuged at 3000 x g for 3 min, and the supernatant stored at -70°C until further use.

7.2.6 ELISAs

To detect IBV antibodies, serum samples were tested using commercial ELISA kits (IDEXX) as described in Chapter 2.10.i.

Immunoglobulin A (IgA) in tears and tracheal washes was assayed using commercial IgA chicken ELISA kit (Abcam, Uk) according to the manufacturer's instructions. In brief, 100 µl of 1:25 dilution of individual tears or tracheal washing was added in the 96-well immunoplates pre-coated with anti-IgA antibodies and incubated for 20 min at room temperature (25°C). After the removal of unbound samples by washing, 100 µl anti-IgA antibodies conjugated with horseradish peroxidase (HRP) was added. Following incubation of the plate for 20 min at room temperature and washing step, the amount of enzyme bound in complex is measured by the addition of 100 µl TMB. Reaction was stopped after 10 min of incubation by adding 100 µl stopping solution and absorbance was measured at 450 nm. The quantity of IgA in the test sample was interpolated from the standard curve constructed from the standards and corrected for sample dilution as per manufacturer's instructions.

7.2.7 Haemagglutination inhibition (HI) test

HI testing was performed using 4 HAU as described in Chapter 2.10.ii. Prior to the HI test, back-titration of the HA antigen was carried out to ascertain the 4 HAU. The HI titres were read as the reciprocal of the highest dilution showing complete inhibition and the HI geometric mean titres were expressed as reciprocal $\log_2$.

7.2.8 Cellular immune responses

7.2.8.1 Analysis of T lymphocyte subsets (CD4+:CD8+) ratio in peripheral blood

To determine the percentage of T-lymphocyte subpopulations, blood was collected from the cephalic vein in heparin tubes (Sigma-Aldrich, UK) at final concentrations of 20 µg/ml of blood, and further diluted (1:1) with RPMI 1640 medium (Sigma-Aldrich, UK). The prepared blood samples (1 ml each) were then over layered onto 0.5 ml of Histopaque-1.077 gradient (Sigma-Aldrich, UK) and centrifuged in 1.5 ml Eppendorf vial at 8000 x g for 90 sec. After centrifugation, the buffy coat formed of mononuclear cells was gently collected, washed twice with a RPMI 1640 medium and adjusted to 1×10^7 cells/ml. The cells were resuspended in 0.5% bovine serum albumin (BSA, Sigma-Aldrich, UK) in PBS (blocking solution) and incubated at room temperature for 15 min. The sample (100 µl) was incubated with antibodies against surface domains of CD4 (mouse anti-chicken CD4-FITC clone CT-4; 0.5mg/ml; Southern Biotech, Birmingham, AL, USA) and CD8 (mouse anti-chicken CD8a-FITC clone CT-8; 0.5mg/ml; Southern Biotech) receptors of T-lymphocytes (antibody final concentrations as 0.2 µl/100 µl of sample) for 30 min in the dark. The stained cells were detected by flow cytometry (BD Accuri® C6, BD Bioscience San Jose, CA, USA) to count the T lymphocytes. The unstained cell sample was used as a negative control to adjust the threshold.

7.2.8.2 Immunohistochemical detection of CD4+, CD8+ and IgA-bearing B-cells in tracheal sections

The trachea samples collected from each bird were immediately placed in aluminium foil cups containing cryo embedding compound (OCT, Solmedia laboratory, Shrewsbury, UK) and frozen in liquid nitrogen (-190°C). The OCT-embedded tracheal samples were cut into 5 µm sections using a pre-cooled cryostat (LEICA CM 1900, Germany), mounted on glass slides (Thermo Scientific, UK) and kept overnight in dark for drying. The sections were fixed in ice-cold acetone for 10 min, air dried at room temperature and stored at -80°C until staining. Staining was carried out as described earlier (Rautenschlein *et al.*, 2011), with some modification. Just prior to staining, slides were removed from -80°C and air dried at room temperature for 10 min. After endogenous peroxidase inhibition using 0.03% hydrogen peroxide (H_2O_2) in PBS for 20 min, the endogenous biotin or biotin-binding proteins in tissue sections were blocked with blocking serum using VECTASTAIN® Elite ABC kit (Vector Laboratories, Burlingame, USA). Following blocking, tissue sections were stained overnight at 4°C in the dark to detect CD4+, CD8+ and IgA+ cells by using mouse monoclonal antibodies to chicken CD4 (clone CT-4; 0.5 mg/ml) and CD8a (clone CT-8; 0.5 mg/ml) at 1:1000, and to chicken IgA (clone A-1; 0.5 mg/ml) at 1:2000. All monoclonal antibodies were procured from Southern Biotech, Birmingham, AL, USA. The colour development was done by addition of 4% 3,3-diaminobenzidine (DAB, VECTOR Laboratories, Burlingame, USA) substrate containing 2% H_2O_2 and 2% buffer stock solution. Sections were then washed in super quality water, and counter stained with hematoxylin (Merck, Germany) for 1 minute, followed by washing in tap water for 5 min. Finally, sections

were dehydrated for 1 min in 96% ethanol, 2 min in 100% ethanol, 3 min in 100% ethanol, 2 min in 100% xylene, 3 min in 100% xylene, 3 min 100% xylene and later mounted with DPX mounting medium and covered with a coverslip. The stained cells were counted in 5 selected microscopic fields at a 400x magnification (fields/trachea). For each sample, the average number of positive cells/400× microscopic field was calculated for each cell type as described previously (Rautenschlein, et al., 2011).

7.2.9 Ciliary protection

At 5 dpc, trachea samples were evaluated according to standard procedure for ciliary movement, and the ciliary protection for each group was calculated. In brief, following euthanasia, trachea was removed aseptically and immediately placed in warm (37^{O}C) TOC medium (Appendix 1). Each trachea was cut into approximately 0.6-mm-thick rings (3 rings from the upper, 4 rings from the middle and 3 rings from the lower) using a tissue chopper (as described in Chapter 2.3.iii) and the tracheal rings were placed in a petri dish containing warm TOC medium. Each ring was examined under a low power microscope (100 x magnifications). Cilia beating in each ring were scored as: All cilia beating = 0, 75% cilia beating = 1, 50% cilia beating = 2, 25% cilia beating = 3, 0% cilia beating = 4. A ciliary activity score of 4 indicates 100% ciliostatsis. According to the European Pharmacopoeia's reference standards, individual birds yielding 80% or more tracheal explants with ciliary activity were considered to have been protected by the vaccine against the challenge virus (Council of Europe, 2007).

Protection scores were calculated according to the following formula:

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

7.2.10 Histopathological evaluation

At 5 dpc, kidneys and tracheas from humanely euthanized birds were collected and fixed in 10% formalin. The tissues were embedded in paraffin wax (50-60ºC) and sections were cut to 7µm thickness. Tissue sections were stained by haematoxylin and eosin (H&E) for microscopic evaluation, the scores attributed according to the criteria described in the Chapter 2.6.ii (Andrade *et al.*, 1982; Chen, et al., 1996).

7.2.11 Real time RT-PCR (RT-qPCR)

RNA was extracted from trachea and kidney samples using the RNeasy Plus Mini Kit following the manufacturer's instructions (Chapter 2.4.i). For quantification of viral RNA quantitative reverse transcription PCR (qRT-PCR) was carried out as described in the Chapter 2.4.v.

7.2.12 Statistical analysis

The comparisons of the means of anti-IBV antibody levels; CD4+:CD8+ ratio in peripheral blood; immunohistochemical detection of CD4+, CD8+ and IgA-bearing B-cells in tracheal sections were performed using one-way analysis of variance (ANOVA), followed by the post-hoc LSD multiple comparison test using GraphPad™ Prism version 6.00 software. Kruskal-Wallis test followed by Dunn's test was used for statistical analysis of the non-parametric RT-qPCR and histopathological evaluation data. Differences were considered significant at $P<0.05$.

7.3 Results

7.3.1 Systemic humoral immune response

7.3.1.1 ELISA

On the day of vaccination, the mean of maternally derived anti-IBV antibody titre was 1750±203. Subsequently, the antibody levels in all three groups declined to below cut-off point (396) by 14 doa. After the booster vaccination with the CR88 at 14 doa, a significant increase in the antibody titres till 28 doa was observed in groups I and II, as shown in Figure 7.1. On these time points, the levels of antibodies were not significantly different between the vaccinated groups (*P<0.05*). After 14 doa though, the antibody titres in group III was always less than the cut-off value of 396 in this assay.

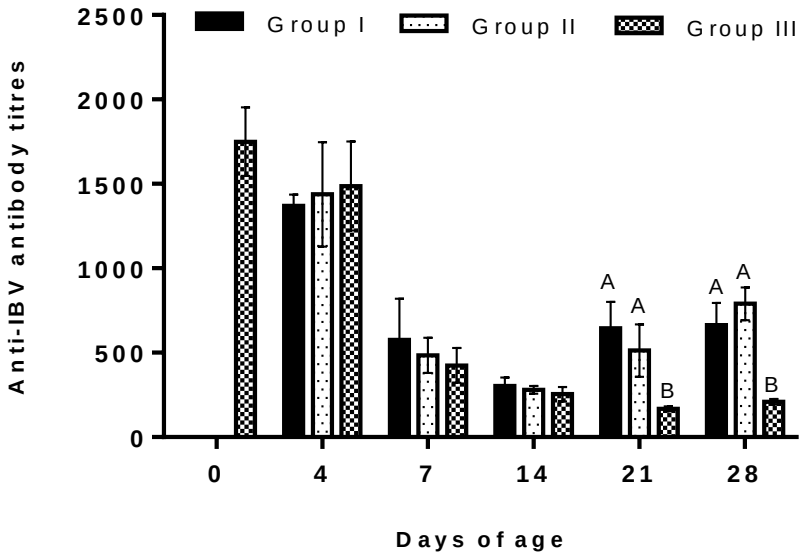

Figure 7.1. Anti-infectious bronchitis virus (IBV) antibody titres of the different groups vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups on those sampling points.

7.3.1.2 HI test

The level of serotype specific antibodies against homologous and heterologous antigens was evaluated by a HI test (Table 7.2). The HI antibody response against all the antigens used showed no significant difference (*P<0.05*) between the groups from 0 to7 doa. However, a lower antibody response was obtained in all groups when the antigen used in the HI test was heterologous (Q1) to the viruses used in the vaccination. On 14 doa, the mean HI antibody titre to the M41 was significantly higher (*P<0.05*) in group II than group I and III. Thereafter, at 28 doa, the levels of antibodies to M41 in group I and II were very similar and significantly higher than group III (*P<0.05*). At 21 doa, group II showed significant increase of HI antibody response against CR88, following revaccination with a homologous antigen. A similar increase was observed in group I on the same sample day. Thereafter, at 28 doa, the HI antibody titre to CR88 antigen was overall significantly higher in group II (log_2 8.2) than that in group I (log_2 4.4) and that in group III (log_2 1.8). At 21 doa, the titres to Q1 in groups I and II were higher compared to group III (*P<0.05*). At 28 doa, the mean HI titre to Q1 was significantly higher (*P<0.05*) in group II than group I with a mean difference of 1.2 log_2.

Table 7.2. Geometric mean anti-IBV HI antibody titre (log_2) in serum of chickens vaccinated with live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Significant differences between the groups (n=5 per group) for each homologous as well as heterologous antigen for each interval are represented by different letters (*P<0.05*)

HI antigen	Groups	Anti-IBV HI antibody titer (log_2) (geometric mean ± standard error) at days of age					
		0	**4**	**7**	**14**	**21**	**28**
M41	Group I	9.2 ± 0.374^{A}	8.2 ± 0.970^{A}	7.4 ± 0.400^{A}	5 ± 0.000^{B}	5 ± 0.548^{A}	4 ± 0.447^{A}
	Group II	9.2 ± 0.374^{A}	8.8 ± 0.374^{A}	7.4 ± 0.400^{A}	6.4 ± 0.510^{A}	5.4 ± 0.400^{A}	4.4 ± 0.400^{A}
	Group III	9.2 ± 0.374^{A}	7.2 ± 0.374^{A}	6.4 ± 0.748^{A}	4.8 ± 0.200^{B}	4 ± 0.632^{A}	2 ± 0.632^{B}
CR88	Group I	8.4 ± 0.400^{A}	7.8 ± 0.374^{A}	6.8 ± 0.490^{A}	5 ± 0.316^{A}	6.8 ± 0.583^{A}	4.4 ± 0.678^{B}
	Group II	8.4 ± 0.400^{A}	8.4 ± 0.245^{A}	6.8 ± 0.374^{A}	5.8 ± 0.200^{A}	7 ± 0.548^{A}	8.2 ± 0.583^{A}
	Group III	8.4 ± 0.400^{A}	7.6 ± 0.400^{A}	7.4 ± 0.245^{A}	4.6 ± 0.600^{A}	3.4 ± 0.245^{B}	1.8 ± 0.490^{C}
Q1	Group I	7 ± 0.316^{A}	3 ± 0.316^{A}	2.4 ± 0.245^{A}	2.2 ± 0.200^{B}	4.4 ± 0.510^{A}	4.4 ± 0.510^{B}
	Group II	7 ± 0.316^{A}	3 ± 0.678^{A}	3.4 ± 0.400^{A}	3.8 ± 0.374^{A}	5 ± 0.548^{A}	5.6 ± 0.245^{A}
	Group III	7 ± 0.316^{A}	3.4 ± 0.316^{A}	2.2 ± 0.200^{A}	2 ± 0.000^{B}	2 ± 0.000^{B}	2 ± 0.000^{C}

7.3.2 Mucosal humoral immune responses

In both, groups I and II, the level of IgA in tears increased significantly *(P<0.05)* compared to control group III from 4 doa, continuing to rise until and initially peaking on 14 doa. In the vaccinated groups, after the second vaccination at day 14, IgA values fell, then increased slightly again through to 28 doa, the day of challenge. The IgA levels in group II were significantly higher *(P<0.05)* than group I from 14 doa until 28 doa, the end of the observation period (Figure 7.2, a).

The level of IgA in tracheal washes in both vaccinated groups was detected from 4 doa, peaking at 7 doa before declining till 28 doa. No significant *(P<0.05)* difference in the level of IgA in tracheal washes induced by the two vaccine groups was observed at any doa (Figure 7.2, b). IgA levels in tears and tracheal washes of both vaccinated groups were significantly higher than the levels from the unvaccinated control group.

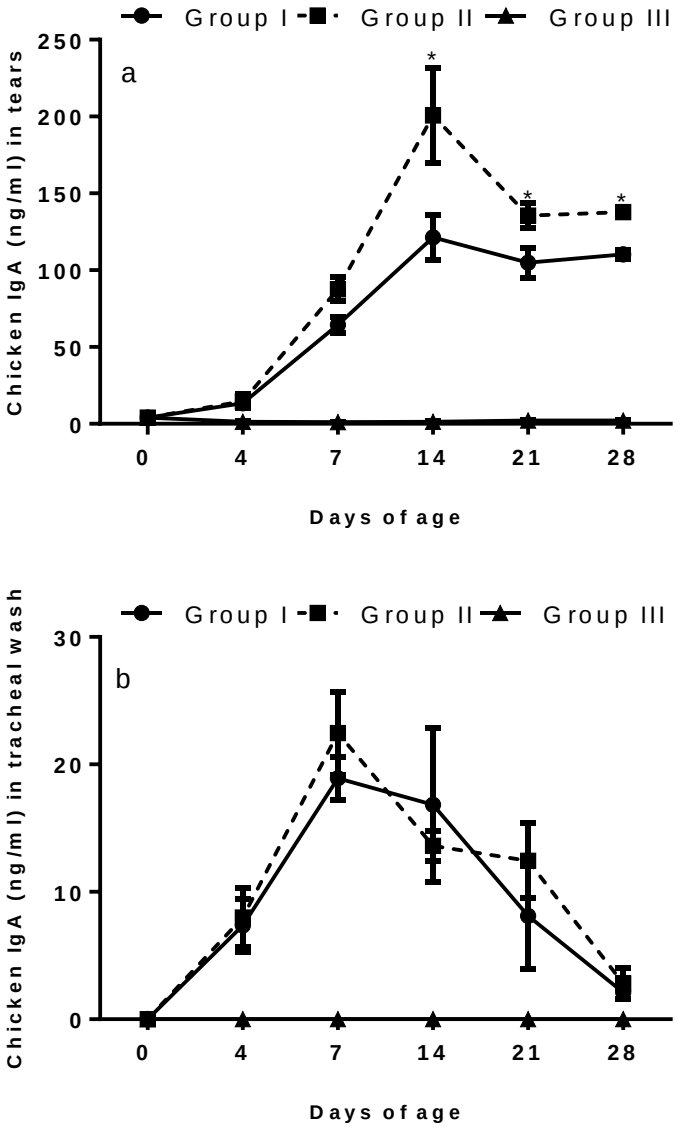

Figure 7.2. Detection of IgA production using ELISA in (a) tears (b) tracheal washes of chickens (n=5 per group) vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. The IgA antibody levels in tears and tracheal wash from control chickens (group III) remained below the detectable level. Asterisks indicate values between the two vaccine groups were significantly different *(P<0.05)* on those time point. Error bars indicate standard error of the mean.

7.3.3 Systemic cell-mediated immune response

7.3.3.1 CD4+:CD8+ ratio in peripheral blood

Flow cytometry results showed that at 7 doa, the CD4+:CD8+ ratios were slightly higher in both vaccinated groups compared to that of the non-vaccinated group, though there was no significant difference (*P<0.05*) between the CD4+:CD8+ ratios of the vaccinated and non-vaccinated groups observed up to 14 doa (Figure 7.3). After the booster vaccination with the CR88 at 14 doa, the ratio of CD4+:CD8+ on 21 doa showed slight increase in both vaccinated groups, being significantly higher (*P<0.05*) in group I compared to that of group II and III. At 28 doa, the ratio was significantly higher (*P<0.05*) in group II compared to groups I and III.

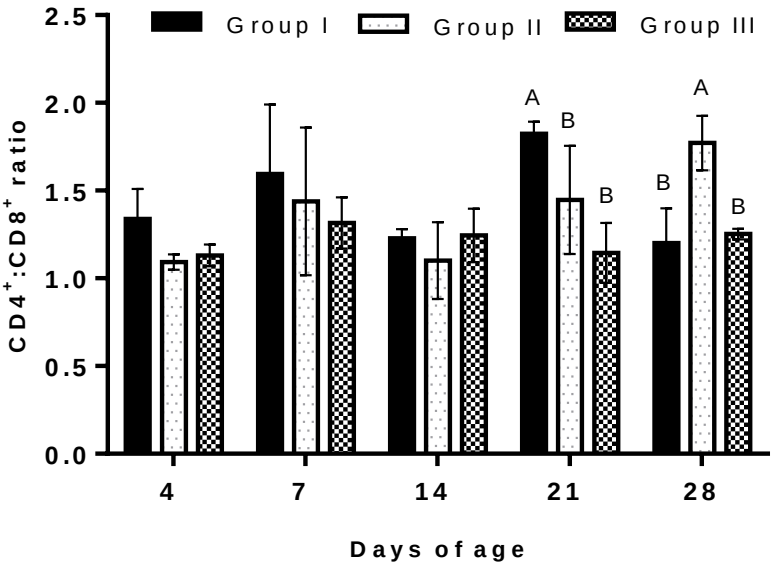

Figure 7.3. The ratio of $CD4^+$:$CD8^+$analyzed by flow cytometry in peripheral blood of chickens vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Depicted are the mean values (n=5 per group) and one standard error. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups on those time point.

7.3.3.2 Mucosal cell-mediated immune responses in the trachea

The kinetics of CD4+, CD8+ and IgA-bearing B lymphocytes in the trachea were studied by IHC (Figure 7.4). The number of CD4+ lymphocytes in the trachea increased significantly (*P<0.05)* from 4 doa in both vaccinated groups compared to the control (Figure 7.5, a). The number of CD4+ cells reached its peak at 4 doa in group I and at 7 doa in group II, before gradually decreasing until 14 doa. After the second immunization, CD4+ cells strongly increased in number by 21 doa in comparison to the non-vaccinated controls before declining again. The difference between the vaccinated groups I and II was not statistically significant (*P<0.05)*. The CD8+ cells subpopulation in groups I and II started to increase significantly (*P<0.05)* at 4 doa, reaching peak at 7 doa and then declining (Figure 7.5, b). After revaccination with CR88 at 14 doa, both vaccinated groups showed a strong increase in the number of CD8+ cells. The number of CD8+ cells were significantly higher in group II than group I at 21 and 28 doa (*P<0.05)*. Overall, the dynamics of the CD8+ cell subpopulations in both vaccinated groups were more dominant than CD4+ cells. At 7 doa, the IgA-bearing B cells increased in vaccinated groups I and II, peaking at 14 doa and showing significant difference compared with the unvaccinated group (*P<0.05)*. The number of IgA-bearing B cells was significantly higher in group II than group I at 21 doa, whereas, no significant (*P<0.05)* difference was observed between both vaccinated groups at 28 doa (Figure 7.5, c).

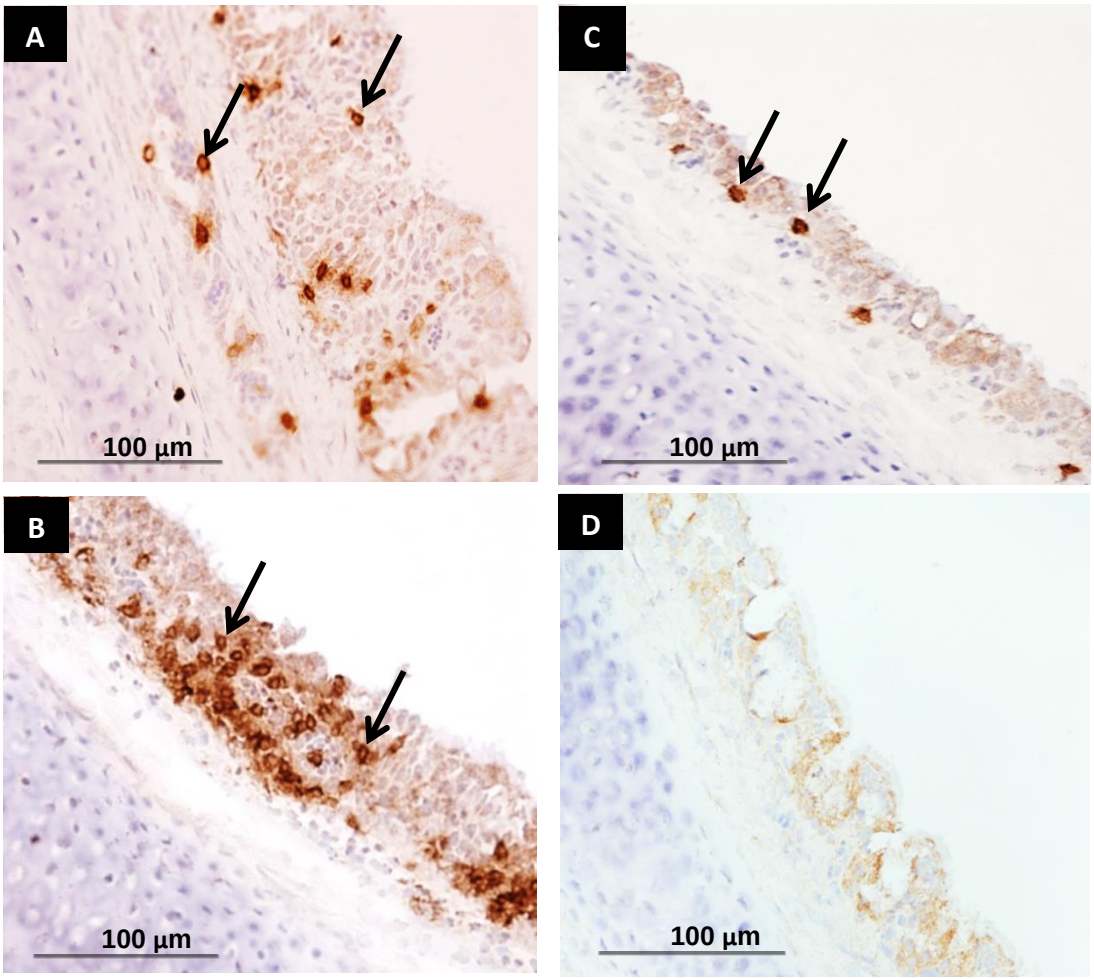

Figure 7.4. Immunohistochemical detection of CD4+ cells in group II at 28 days of age (A), CD8+ cells in group II at 28 days of age (B), IgA-bearing B-cells group II at 28 days of age (C), in tracheas of chickens vaccinated with live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control (D). Magnification (400x). Arrows indicate positive cells.

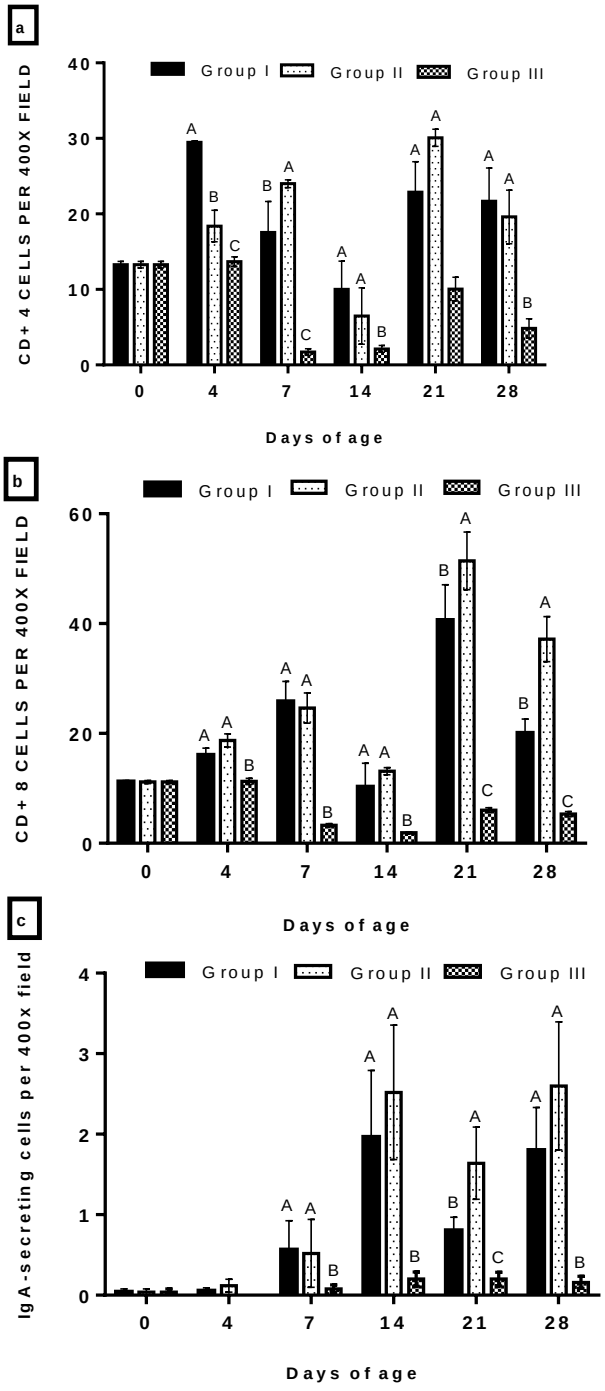

Figure 7.5. Summary of CD4+ cells (a), CD8+ cells (b), and IgA-secreting cells (c) determined by immunohistochemical staining in the trachea of chickens vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) was kept as control. Depicted are the mean values (n=5 per group) and one standard error. Where values were significantly *(P<0.05)* different these are shown with different letters and all other values were not significantly *(P<0.05)* different between the groups at those time points

7.3.4 Protection

After challenge, no clinical signs were observed in either vaccinated groups. In the unvaccinated group, respiratory signs including as coughing, sneezing, head shaking, tracheal rales and nasal discharge were observed until 5 dpc. The highest percentage of ciliary protection (97%) was observed in group II, followed by group I (89.75%). The unvaccinated challenged group (group III) showed little protection (12%) compared to the vaccinated challenged groups.

Viral RNA loads, in all tracheal samples collected, were significantly higher (*P<0.05)* (4.416 log REU RNA) in the unvaccinated challenged group (III) compared to the vaccinated groups (I and II) as measured by real time RT-qPCR, at 5 dpc. The vaccinated groups, I and II showed mean log REU of viral RNA of 1.016 and 0.555, respectively, with no significant difference between these groups (Figure 7.6). Overall viral RNA in the kidney samples of all the groups was low compared to tracheal samples. The viral RNA load in kidneys in group III was significantly higher (*P<0.05)* than in group II, whereas, group I showed no significant difference (*P<0.05)* in log REU of viral RNA with either of group II and III.

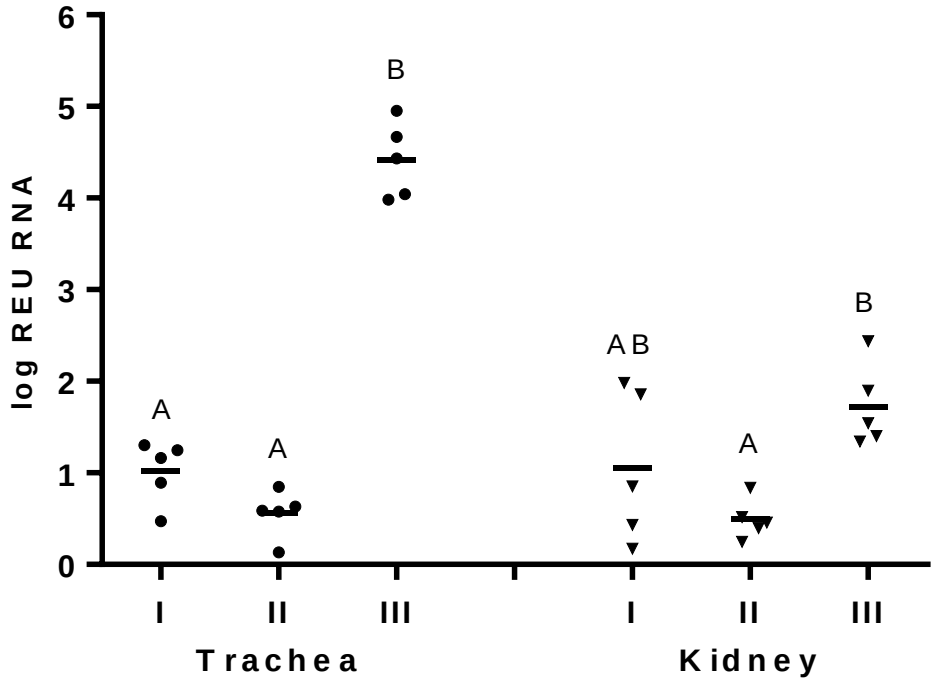

Figure 7.6. Quantification of infectious bronchitis virus (IBV) expressed as log REU of RNA, in trachea and kidney measured by real time RT-PCR after 5 dpc from chickens experimentally challenged at 28 days of age with Q1 strain of IBV (n=10 per group). The chickens were previously vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) kept as control received sterile water. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test indicated with different letters (*P<0.05).*

7.3.5 Histopathology

Histopathological lesions in tracheas and kidneys were induced by challenge virus in all the groups at 5 dpc. Marked histopathological changes occurred in group III (non-vaccinated group) with mean scores of 10.2 and showed significant difference with group II (*P<0.05)*, but not with group I (Figure 7.7). The mean lesion scores for kidneys in group III was significantly higher (*P<0.05)* than group II, whereas, group I showed no significant difference (*P<0.05)* in mean lesion scores with either group II and III. However, overall mean lesion scores in kidneys were low compared to mean tracheal lesion scores.

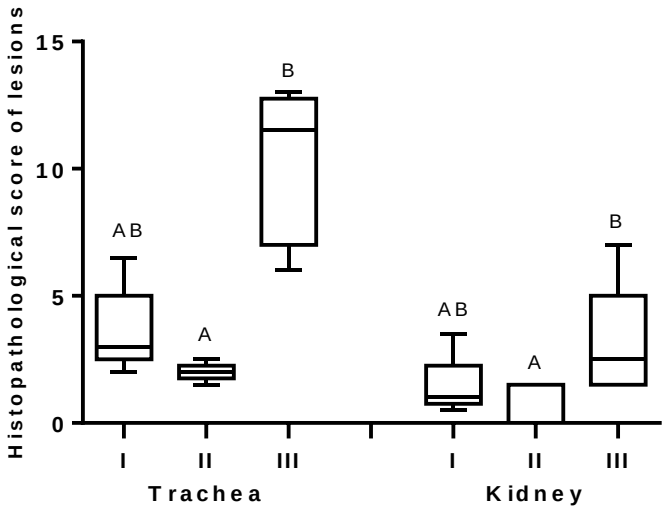

Figure 7.7. Means of histopathological scores of lesions in trachea and kidney samples after 5 dpc from chickens experimentally challenged at 28 days of age with Q1 strain of infectious bronchitis virus (n=10 per group). The chickens were previously vaccinated with a live H120 alone (group I) or in combination with CR88 (group II) at day-one. Both groups were again vaccinated with CR88 at 14 days of age. One group (group III) kept as control was inoculated with sterile water. Significant differences between the groups were detected by Kruskal-Walis test followed by Dunn's mean test indicated with different letters (*P<0.05).*

7.4 Discussion

The vaccination regime, chosen in this study for group I, is based on the research demonstrating that improved protection was seen when two vaccines used were of different serotypes (Awad, et al., 2015; Cook JKA, 1999; Terregino, Toffan, Beato, et al., 2008). These researchers also emphasized that the vaccination programme used in their experiments may not protect the respiratory tract against challenge with every new IBV serotype to emerge. It is also evident that despite the use of Mass type vaccine at day 0, followed by 793B type vaccine at 14 doa (same as in group 1 in this study), significant number of new IBVs are still emerging under field conditions e.g. QX, IS/885/00, IS/1494/06 and most recently Q1. Therefore, in order to optimize the use of currently available vaccines, to achieve better immunity and to assess protection against newly emerged Q1 strain, the vaccination regime for group II was also included in this work.

At 1-day-old, chicks had high ELISA anti-IBV antibody titres in all groups, which declined and dropped to below the cut-off point by 14 doa. In the groups that received the vaccine at one day old, these low antibody levels could result from the partial neutralization of the vaccine virus in the target tissues by the maternal antibodies present in the broilers at that age, with a consequently low replication of the vaccine virus and poor stimulation of the humoral response (Davelaar & Kouwenhoven, 1977; Mondal & Naqi, 2001). Later, after the second vaccination at 14 doa, an increase in the antibody titres was observed until 28 doa (day of challenge) in groups I and II with no significant difference in antibody levels between these vaccinated groups ($P<0.05$). HI antibody levels declined by 14 doa against the homologous and heterologous virus antigens, showing similar patterns

to declining ELISA titres. Interestingly, by 28 doa, there was no significant difference between vaccinated groups I and II in terms of the level of antibodies to M41, whereas, the HI titres to 793B and Q1 were significantly higher in group II than group I (*P<0.05*). The role of antibody in the control of IBV infection remains controversial as workers have shown that circulating antibody titres did not correlate with protection from IBV infection (Gelb, et al., 1998; Gough & Alexander, 1979; Raggi & Lee, 1965). However, other studies demonstrated the importance of humoral immunity in disease recovery and virus clearance (Thompson *et al.*, 1997; Toro & Fernandez, 1994). In our study, as expected, the higher HI titres were obtained using antigen homologous to vaccine strains. However, the chicks also appeared to be protected against heterologous challenge. This could be due to the presence of local immunity of the upper respiratory tract, induced by vaccination thus reducing the replication of challenge virus.

The role of IgA antibodies is important for mucosal immunity to IBV and its presence in tears following IBV antigen inoculation has been reported earlier (Cook *et al.*, 1992; Toro & Fernandez, 1994). In this study, a gradual increase in IgA levels was observed in tears for both vaccinated groups during the first two weeks after vaccination. These results are in agreement with previous research reporting similar kinetics of lachrymal fluid IgA production to H120 vaccination (Okino, et al., 2013; Toro *et al.*, 1997). In addition, after the second vaccination, lachrymal IgA levels decreased in both vaccinated groups, though, the levels in group II remained significantly higher *(P<0.05)* than group I. This observation may indicate a decrease of lachrymal IgA levels after the second vaccination is most likely due to partial neutralization of the anti-IBV IgA. A sharp decrease of IgA-IBV in vaccinated chicks

was also observed after challenge (Davelaar, et al., 1982). In addition, no significant rise in specific lachrymal IgA of vaccinated chickens was detected after subsequent challenge with Ark-IBV isolate, explaining the probable role of neutralizing antibodies in the lachrymal fluid at the time of challenge (Joiner, et al., 2007).

IBV-specific IgA can also be found in tracheal washes after an infection with strain of IBV M41 (Cook, et al., 1992; Thompson, et al., 1997). In this study, the pattern of IgA in tracheal washes in both vaccinated groups I and II was closely parallel, reaching peak at 7 doa and thereafter, declining till 28 doa, suggesting a short duration of the local humoral immunity in the trachea. Although there have been conflicting reports on the relative concentrations of IgA in the avian respiratory tract (Chhabra & Goel, 1980; Gillette, 1981; Holmes, 1973), our results are consistent with Hawkes *et al* (1983), which showed IgA antibodies in tracheal washes only at day 7 after vaccination (Hawkes, et al., 1983). Interestingly, in both vaccinated groups, the second vaccination did not cause any rise in tracheal IgA level. Similar findings have also been reported, revealing that the revaccination with homologous IBV (M41 or H strains) (Gillette, 1981), and secondary M41 IBV exposure (Thompson, et al., 1997), did not induce the secondary secretary antibody response in tracheobronchial washings.

Consistent with the notion that CMI is protective against IBV (Chubb *et al.*, 1988; Seo, et al., 2000), we next sought to study the level of systemic and local cellular immune responses. CD4+ cells may directly produce antiviral cytokines, which increases B cell activity and promotes the proliferation, maturation, and functional activity of CD8+ CTLs, thus playing a critical role in controlling IBV infection (Raj & Jones, 1997a; Seo & Collisson, 1998). The ratio of CD4+:CD8+ has been widely

shown to be indicative of the general immune system status (Chen *et al.*, 2011; Dalgaard *et al.*, 2010). In this study, the CD4+:CD8+ ratio showed no significant (*P<0.05*) variation among the groups till 14 doa. Nevertheless, the ratio at 28 doa was found significantly higher (*P<0.05*) in group II than in groups I and III indicating that second vaccination at 14 day-old in group II has probably enhanced the cellular immunity by promoting the differentiation and proliferation of CD4+ cells in peripheral blood. There are no specific data regarding the effects of different IBV vaccination on CD4+:CD8+ ratio in peripheral blood so as to compare the present findings, however, Yohannes *et al.* (2012) have reported significantly (*P<0.05*) higher CD4+:CD8+ ratio in IBV infected chicks than in the controls (Yohannes *et al.*, 2012). In addition, the high CD4+:CD8+ ratio has also been associated with increased humoral incompetence in chickens, as a low CD4+:CD8+ ratio and a reduced response against sheep red blood cells have been reported earlier (Dunnington EA, 1992). In this study, significantly (*P<0.05*) higher HI titres at 28 doa to 793B and Q1 in group II than those in group I may be attributed to the high CD4+:CD8+ ratio in group II at that time point. However, the significance of this in relation to protection remains to be determined.

The results of IHC in tracheal tissue showed that the number of CD4+ lymphocytes started increasing from 4 doa in both vaccinated groups, as compared to the control. At 28 doa, no significant difference was reported between the vaccinated groups. The CD8+ cells subpopulation in both vaccinated groups started to increase significantly (P<0.05) at 4 doa, reaching peak at 7 doa and then declining in number until 14 doa, suggesting that infiltration and recruitment of these cells occurs in the first two week of initial IBV vaccination. Similar to the findings of the present study,

in the trachea, CD8+ cells recruitment in response to infection were at a maximum by 7 days post infection (dpi) and CD4+ cells were not recruited until 5 dpi. This was reported by Raj & Jones (1996a). This work also showed an overall higher infiltration of CD8+ cells in numbers compared to CD4+ cells in both vaccinated groups. This observation is consistent with a previous study (Raj & Jones, 1996a), where CD8+ cells were also found to predominate compared to CD4+ cells in trachea after IBV infection. Moreover, the current study also documents significantly higher number of CD8+ cells in vaccinated chicks of group II compared to group I on 21 and 28 doa, respectively. The IgA-bearing B cells in vaccinated groups reached peak at 14 doa, however, the number of these cells were significantly higher in group II in comparison to group I at 21 and 28 doa (*P<0.05)*. This pattern of recruitment of B cells later than either class of T cells is in accordance with earlier study (Janse, et al., 1994) who contended that local immunity against IBV is mediated mainly by T-cells.

In this study, following the Q1 challenge, ciliary protection was higher in group II, vaccinated with mixed H120 and CR88 vaccines at day-old, than in group I, vaccinated at day old with H120 alone. Furthermore, the results of RT-qPCR showed that the viral RNA load at 5 dpc in the trachea, was higher in group I than group II although the difference was not statistically significant (*P<0.05*). In agreement with this, the scores of histopathology in the trachea showed that the damage caused by the Q1 was higher in group I than II and showed no significant difference in mean lesion scores with either of group II and III. On the basis of these tracheal histopathological assessments, chickens in group II were better protected

compared with those in group I and this better protection might be attributed by various factors including those discussed below.

Although the anti-IBV ELISA antibody titre results indicated that there was no significant difference between the two vaccinated groups at the day of challenge, group II showed higher ciliary protection than group I. This observation is consistent with previous studies which have shown that circulating antibody levels were of minor importance in the protection of the respiratory mucosa against IBV challenge (Holmes, 1973; Ignjatovic & Galli, 1994).

From our results, it appears that such overall higher protection could be due to significantly higher levels of CD8+ cells in the tracheal tissues in group II than group I at day of challenge. Previous study has shown that CD8+ cells are important contributors to viral clearance in respiratory virus infections, utilizing contact-dependent effector functions, IFN-γ and tumour necrosis factor-α (Bruder *et al.*, 2006). Therefore, we may speculate that the group II's higher CD8+ cell reaction than the group I could have contributed to the faster viral clearance after challenge with Q1, explaining the differences between the vaccinated groups in their tracheal protection. This possible explanation agrees with other studies that emphasized the involvement of local CD8+ cells in the infection of chickens with respiratory pathogens, such as Newcastle disease virus (Russell *et al.*, 1997) and *Mycoplasma gallisepticum* (Javed *et al.*, 2005). Additionally, group II's higher levels of IgA in lachrymal fluid, compared to group I, could reduce the tracheal histopathological damage which also corroborates the hypothesis that the traditional role of IgA is to prevent pathogen entry at mucosal surfaces and neutralize virus in infected epithelial cells (Lamm, 1997). IBV-specific IgA antibodies

in lachrymal fluid were correlated with resistance to IBV reinfection (Cook, et al., 1992; Davelaar, et al., 1982; Toro & Fernandez, 1994). Our results are in agreement with a recent study by Okino *et al* (2013), in which the authors concluded that IBV IgA antibodies in lachrymal secretions and the expression of granzyme-A and CD8 genes in tracheal tissues after H120 vaccination, provides a reliable approach to monitor immune protection status in the trachea, as shown by examination for ciliostatsis, histopathology and viral replication (Okino, et al., 2013). For our study, we aimed to stain for a variety of cell-surface markers and thereby identify the T cell populations infiltrating the trachea. This provides further information about the role of cell-mediated immunity in protection given by different live IBV vaccination regimes against a novel IBV Q1 challenge.

The results of RT-qPCR and scores of histopathology in the kidneys showed that the damage caused by the Q1 was higher in group I than II, and showed no significant difference in the mean lesion scores with either of group II and III. Specific cytotoxic T lymphocytes have been shown to be important for the systemic clearance of nephropathogenic IBV and reduction of kidney lesions (Collisson, et al., 2000). A plausible explanation is that a higher CD8+ cells response in the tracheal tissues (the portal of entry of challenge virus) in group II compared to group I could have prevented the challenge virus becoming viraemic thus failing to reach the kidneys. This provided an efficient prevention of kidney infection, as measured by viral RNA load and histopathological lesion scores in renal tissue.

In conclusion, chicks vaccinated with H120 and CR88 at day-old, followed by CR88 at 14 doa, showed significantly higher CD8+ responses in the trachea and higher lachrymal IgA levels compared to those vaccinated with H120 alone. In terms of

ciliary protection against Q1, though both vaccinated groups were protected, the combined vaccination of H120 and CR88 of day-old chicks, followed by CR88 at 14 doa, showed higher ciliary protection and less RNA load in trachea and kidneys, wherein histopathological lesions are reduced. This study highlighted the potential modulation of chick immune response with the use of currently available live vaccines so that better protection against variant IBVs can be afforded.

Chapter 8: General discussion and future work

Avian infectious bronchitis virus (IBV) continues to cause serious economic losses in chicken production globally. Vaccination has been considered as the most reliable approach for controlling IBV infection (Meeusen, et al., 2007). However, current vaccines have proved to be poor in controlling IBV especially when new variant viruses have emerged (De Wit, 2000; de Wit, et al., 2011). Concurrent circulation of both classic and variant IBVs have been identified in most parts of the world, raising major challenges to global prevention and control efforts. During the past few decades there have been reports of new virulent field isolates that represent variant strains and the field cases reported until now suggest that these variants have wide tissue tropism and high pathogenicity (Gelb, Lunt, et al., 1991; Jackwood, 2012; Shaw, et al., 1996; Zanella, et al., 2000). Though the mechanisms behind difference in susceptibility of chickens to infection by different IBVs have been studied and hypothesized extensively, but these are still not well understood.

The first two studies (Chapter 3 and 4) involved the use of *in vitro and ex vivo* systems to study the host determinants of IBV pathogenicity. The role of apoptosis and innate immune responses was investigated in chicken embryo kidney (CEK) cells and tracheal organ cultures (TOCs) following infection with IS/885/00-like, QX-like and M41 IBV strains. We noted that nephropathogenic IBV strains 885 and QX induced greater apoptosis in CEK cells than the respiratory tropic M41, whereas M41 induced higher apoptosis in TOCs than 885 and QX. Furthermore, compared to M41, 885 and QX caused greater induction of toll like receptor 3 (TLR3), melanoma differentiation associated protein 5 (MDA5) and interferon beta (IFN-β) in CEK cells. In contrast, M41 infection caused higher induction of these three genes than 885 or QX in TOCs. From these studies we concluded that higher apoptosis together with

elevated levels of TLR3, MDA5 and IFN-β expression correlates with pathogenicity of IBV strains in kidney and trachea. A recent study showed that MDA5, but not TLR3, is involved in the sensing of IBV (Kint, et al., 2015). It is possible that this difference may have been due to variation in the MOI used and origin of cells/TOCs. However, further work is required to understand the precise role of MDA5 and TLR3 in host defence against IBV infection and/or disease pathogenesis. More comprehensive work needs to be done to paint a clearer picture of how IBV regulates apoptosis during infection, since recent reports have suggested the possible activation of more than one apoptotic pathway during infection.

Our findings from above studies raised a tantalizing possibility of using CEKs and TOCs to rapidly predict the tissue tropism and virulence of novel IBV strains. In spite of advantage of using *in vitro* model is that explants of the same animals can be used to compare different viral strains, for corroborating the *in vitro* results, the *in vivo* work (Chapter 5) was undertaken. In this study we investigated the differential immunopathogenesis in chickens infected with the three IBVs used in the above studies. It was confirmed that the histopathological changes, proinflammatory and innate immune gene response could be induced on a diverse scale depending on the IBV strain. Our results showed that greater upregulation of expression of proinflammatory cytokines (IL-6, IL-1β and LITAF) and innate immune response genes (TLR3, MDA5 and IFN-β) was induced by M41 in trachea and 885 and QX in kidney. Moreover, we observed that mRNA levels of these genes correlated with the pattern of histopathological lesion scores in trachea and kidney produced by IBV strains used in this study. This has also been supported by a more recent study (Okino, et al., 2014) in which upregulation of expression of proinflammatory

cytokines such as IL-6, IL-1β, induced by the M41 strain of IBV was found to be partially associated in the pathological alterations in trachea of non-immune challenged chickens. Parallel with our findings, previous studies also reported a significant increase of TLR3 mRNA expression in trachea of IBV infected chickens when compared to the uninfected controls (Kameka, et al., 2014; Wang, et al., 2006). Also MDA5 mRNA expression levels were reported to be significantly increased in chicken kidney tissue after nephropathogenic IBV infection suggesting a role of chicken MDA5 against IBV infection (Cong, et al., 2013).

The present results about correlation of innate immune response genes (TLR3, MDA5 and IFN-β) expression with the tracheal and kidney pathology in chickens infected with different IBVs further confirm our *in vitro* work findings. In spite of our work on the evaluation of differential modulation of PRRs (TLR and MDA5), type I IFNs, and pro-inflammatory cytokine mRNA expression in chickens in response to different virulent IBV strain, analysis of expression of these molecules may not be sufficient to completely understand the IBV immunologic mechanisms. Further studies may employ these findings to understand the underlying mechanism of natural host's response to different attenuated IBV strains as well as in context of vaccine induced immune responses and protection against challenge with virulent IBVs. For almost two decades IBV 793B has been associated with disease and for most of that period one or more live 793B vaccines have been in use (Cook, et al., 1996; Martin, et al., 2014). The persistence of such 793B is of concern and challenges our ability to both diagnose and control it. Therefore, better understanding of immunopathogenesis needs to be developed for an effective control of diseases caused by virus. In chapter 6 we comprehensively

evaluated the immunopathogenesis after 793B infection either *in vitro* or *in vivo*. It has been found that *in vitro* higher apoptosis together with elevated levels of TLR3, MDA5 and IFN-β expression correlated with *in vivo* pathogenicity of 793B in trachea and kidney. In addition, the higher upregulation of expression of proinflammatory cytokines (such as IL-6 and IL-1β) and LITAF in trachea and kidney of SPF chicks coincided with the highest viral loads and microscopic tracheal lesions, indicating a role for both of these cytokines with high virus loads and the development of lesions in these organs. In contrary, most recent studies showed that MDA5, but not TLR3, is involved in the sensing of IBV (Kint, et al., 2015). Furthermore, no significant changes in TLR3 expression were observed in kidney following infection with attenuated CK/CH/LHLJ/04V strain, which could be due to the use of different strains of virus (Xu, et al., 2015). Future studies should be performed to further characterize the role of TLR3 during the IBV infection in chickens.

In the past two decades the majority of the serotypes/genotypes of IBV that have emerged appear to be endemic (Bochkov, et al., 2006) and this constant emergence of variant strains of virus poses a serious threat to health, production and welfare of birds. IBV genotype, Q1, which is genetically and serologically distinct from the classical IBVs, was first time reported in China (Yu, et al., 2001). Since its identification, Q1 has been reported from many parts of world such as the Middle East (Ababneh, et al., 2012), Europe (Toffan, et al., 2011) and South America (Jackwood, 2012). To contain this strain, an effective vaccination programme is needed. However, very little is known about the cross protection induced by the commercially available vaccines or vaccination regimes against this variant Q1. In addition, the kinetics of, and the relationship between local and systemic humoral

and cell mediated immune responses induced by different IBV vaccination regimes, needs to be better understood for protection against emerging IBV strains. Therefore, in Chapter 7, day-old broiler chicks were vaccinated with live H120 alone (Group I) or in combination with CR88 (Group II). Both groups were again vaccinated with CR88 at 14 days of age (doa). One group was kept as the control (Group III). At 28 doa, all the groups were challenged with Q1.

After the second vaccination at 14 doa, an increase in the antibody titres using ELISA was observed until 28 doa (day of challenge) in groups I and II with no significant difference in antibody levels between these vaccinated groups ($P<0.05$).

The cytotoxic T cell response in the tracheal tissues and level of IgA in lachrymal fluid were significantly higher in the group vaccinated with H120 and CR88 at day-old followed by CR88 at 14 doa (Group II) in comparison to the group vaccinated with H120 alone at day-old followed by CR88 at 14 doa (Group I). Combined vaccination of H120 and CR88 of day-old chicks followed by CR88 at 14 doa (Group II) showed higher protection against Q1 owing to higher cytotoxic T cell response in the tracheal tissues and a high level of IgA in lachrymal fluid. This study shows the benefits of assessing local and cellular immune response besides serum antibodies in IBV vaccination-challenge studies. Taking the above into account, the future studies should be performed on the activity of CMI, such as the measuring the cytotoxic mechanism of CTLs. In addition, our study confirms the suitability of measuring the viral load in trachea and kidney by qRT-PCR for evaluating the efficacy of vaccines by challenge infection.

Essentially, the findings of the studies presented here shed some light on the importance of *in vitro* and *in vivo* comparison of virulence, immunopathogenesis and protection study involving variant IBVs. The data set we have produced in chapters 5 and 6 were obtained from SPF chicks without maternally derived antibodies. These data may have further implications for a better understanding of IBV immunopathogenesis if further in-depth studies are undertaken using commercial broilers and SPF chicks with maternally derived antibodies. Our results have been published in scientific journal and presented in national and international conferences.

In spite of the novel aspect our work on the evaluation of differential modulation innate immune mediators in response to IBV infection *in vivo* as well as *in vitro*, analysis of expression of these molecules may not allow complete understanding of IBV immunological mechanisms. One major characteristic of IBV virions is the high variability of the S1 subunit resulting in the occurrence of new strains. Most serotypes differ as much as by 20-25% from each other in their S1 sequence but some serotypes show a difference of 50% (Cavanagh, 2007). Because of the high diversity in the different S1 sequences, understanding the molecular mechanism of IBV pathogenicity and the relation of IBV to host is a key point in IBV control. Further studies are needed to identify the role of these viral proteins responsible for mediating the differential host innate responses against different IBVs and the cellular source of innate mediators produced in the target organs.

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Appendix I

(Reagents and protocols)

Phosphate buffered saline (PBS)

NaCl	85.0 g
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	10.7 g
Sodium dihydrogen orthophosphate ($NaH_2PO4.2H_2O$)	3.90 g

Made up to 1 litre with sterile distilled water

0.02% EDTA (Ethylenedinitrilo)-tetraacetic acid, Dipotassium salt) in PBS

EDTA	0.1 g
PBS	500 ml

Trypsin solution 0.25%

0.02% EDTA	90 ml
Trypsin[54] 2.5%	10 ml

Stock antibiotic solution

Crystapen[55]	600 mg
Streptomycin sulphate BP [56]	1.0 g
Sterile distilled water	4 ml

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

[54] Life Technology, UK

[55] Glaxo, Greenford, UK

[56] Evans Medical, Greenford, UK

Growth medium for CEK cells

10x Minimum Essential Medium-Eagles[57]	100 ml
7.5% Sodium bicarbonate[58]	20 ml
Penicillin-streptomycin solution	4 ml
L-glutamine 200 mM[59]	
10 ml	
Fetal bovine serum[60]	5 ml
Sterile distilled water	to make 1000 ml

TOC culture medium

10x Minimum Essential Medium-Eagles [61]	100 ml
7.5% sodium bicarbonate	20 ml
Sterile distilled water	900 ml
Penicillin-streptomycin solution	4 ml

HEPES buffer

HEPES [62]	2.95 g
NaCl	4.09 g
$CaCl_2$	0.08 g

Add sterile distilled water to 500ml distilled water, pH adjusted to 6.5 with 1N NaOH

[57] Life Technology, UK

58 Life Technology, UK

59 Sigma-Aldrich, UK

60 Sigma-Aldrich, UK

61 Life Technologies, UK

62 Life Technologies, UK

Tris-buffer saline (TBS)

Tris[63]	6.05 g
NaCl	8.76 g
Sterile distilled water	800 ml

Adjust pH to 7.5 with 1M HCl and make volume to 1000 ml using distilled water

cDNA synthesis reaction mixture I[64]

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

cDNA synthesis mix[65]

10X RT buffer	2 µl
25 mM $MgCl_2$	4 µl
0.1 M DTT	2 µl
RNasrOUT (40U/µl)	1 µl
Superscript III (200 U/µl)	1 µl

[63] Sigma-Aldrich, UK

64 Invitrogen, Paisley, Scotland

65 Invitrogen, Paisley, Scotland

Quantitative reverse transcription PCR (qRT-PCR) reaction mixture

2x Rotor Gene probe[66]	12.5 µl
IBVRT1	2 µl
IBVRT2	2 µl
IBVRT3 TaqMan® probe	0.5 µl
Rotor-Gene RT Mix[67]	0.25 µl
RNase-free water	5.75 µl

10x Tris-borate-EDTA (TBE)[68]

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

Oligos[69] for RT-PCR

Oligos received were reconstituted as per manufacturer's instructions. The stock oligos were diluted to 1:10 to make working solutions

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

66 Qiagen, UK

67 Qiagen, UK

68 Sigma-Aldrich, UK

69 Invitrogen, Paisley, Scotland

10mM dNTP's Working Solution

dATP, dCTP, dGTP, dTTP at 100mM[70]

Take 20µl of each dNTP, total volume 80µl

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

RT-PCR reaction mixture

5X Buffer[71]	1 µl
DTT	0.5 µl
dNTP	0.25 µl
Ultra-pure Water	2.13 µl
RNase inhibitor	0.12 µl
Superscript II (200 U/ µl)[72]	0.25 µl
Negative Oligo (10 pmoles/µl) (SX2-)	0.75 µl

Nested PCR 1 reaction

PCR Supermix [73]	19 µl
SX1+	0.5 µl
SX 2-	0.5 µl

Nested PCR 2 reaction

PCR Supermix	23.5 µl
SX3+	0.5 µl
SX 4-	0.5 µl

[70] Invitrogen, Paisley, Scotland

71 Invitrogen, Paisley, Scotland

72 Invitrogen, Paisley, Scotland

73 Invitrogen, Paisley, Scotland

Loading Buffer

Ficol	3 g
1XTBE	20 ml

Gel preparation

1.5% agarose[74]	0.58 g
TBE	35 ml

4% Paraformaldehyde (PFA)

For 250 ml:

1. Weigh out 10 g Paraformaldehyde (wear mask) into suitable bottle/flask
2. Add approximately 200 ml 1xPBS
3. Heat solution on stirring hotplate to 60°C (do in fume hood as noxious fumes), if solution has not fully dissolved add a few drops of 1M NaOH (solution won't dissolve if not at correct pH)
4. Cool solution, check pH is 7.2-7.4
5. Top up to final volume of 250 ml with 1x PBS
6. 4% PFA can be stored for up to 1 month, room temperature

[74] Sigma-Aldrich, UK

RNeasy® Mini Kit for total RNA extraction

Before start

- Equilibrate all buffers to room temperature
- Remove tissues from RNAlater® to stabilize
- Add 10 µl of β-mercaptoethanol (β-ME) to 10 ml of RLT buffer (can be stored at room temperature up to 1 month)
- Add 4 volumes of ethanol (96-100%) to RPE buffer to make a working solution.

Protocol

1. To the harvested cells or tissues (≤30 mg), add 600 µl of prepared RLT buffer containing β-ME into 2 ml Eppendorf tube
2. Disrupt and homogenize the tissue using stainless steel beads in a TissueLyser[75]
3. Centrifuge the lysate at 13000 rpm for 3 min, carefully remove the supernatant into a clear 1.5 ml Eppendorf tubes
4. To this, add 1 volume of 70% ethanol and mix well by pipetting
5. Transfer up to 700 µl of the sample, including any precipitate, to an RNeasy Mini spin column placed in 2 ml collection tube
6. Centrifuge at 8000 rpm for 15 s, discard flow through
7. Add 700 µl of buffer RW1 to the spin column
8. Centrifuge at 8000 rpm for 15 s, discard flow through
9. Add 500 µl of buffer RPE (working solution) to the spin column

[75] QIAGEN, UK

10. Centrifuge at 8000 rpm for 15 s, discard flow through
11. Add 500 µl of buffer RPE (working solution) to the spin column
12. Centrifuge at 8000 rpm for 2 min, discard flow through
13. Place the spin column in a new 1.5 ml collection tube and add 30 µl of RNase-free water directly to the spin column
14. Centrifuge at 8000 rpm for 1 min to elute the RNA
15. Store RNA at -70^{o}C

QlAamp viral RNA extraction

Equilibrate samples and buffers to RT

Before start

- Equilibrate samples and all buffers to room temperature
- Add 310 µl Buffer AVE into the tube containing 310 µg lyophilized carrier RNA
- Mix the carrier RNA completely
- For extracting RNA from 55 samples, add the 310 µl reconstituted carrier RNA to 31ml buffer AVL (stable at refrigeration temperature for 48 hours)
- Before use of buffer AVL, look out for precipitation. If there is any precipitation, heat the buffer AVL at 80^{o}C for no more than 5 min (but not more than 6 times)

Protocol

1. To 140 µl of sample, add 560 µl of buffer AVL containing carrier RNA into 1.5 ml Eppendorf tube
2. Vortex for 15 sec
3. Incubate at room temperature for 10 min
4. Pulse centrifuge
5. Add 560 µl of 100% ethanol
6. Vortex for 15 sec
7. Pulse centrifuge
8. Label the spin columns

9. Carefully transfer 630 µl from tube into spin column
10. Centrifuge at 8000 rpm for 1 min, discard flow through
11. Place spin column into a clean collection tube and add 500 µl of buffer AW1
12. Centrifuge at 8000 rpm for 1min, discard flow through
13. Place the spin column into a clean collection tube add 500 µl of buffer AW2
14. Centrifuge at 13,000 rpm for 3 min, discard flow through
15. Place the spin column into a clean 1.5 ml Eppendorf tube and add 60 µl of RNase-free water
16. Incubate the spin column at room temperature for 1 min
17. Centrifuge at 8000 rpm for 1min to elute the RNA
18. Store RNA at -70^{o}C

Haemagglutination inhibition test

HA test for preparation of 4 HA antigen

1. Add 25 µl of PBS to each well of a U-bottom micro-titre plate.
2. Add 25 µl of virus antigen to the first well.
3. Make two fold dilutions of 25 µl of the virus antigen across the plate, discard final 25 µl.
4. Add a further 25 µl of PBS to each well (total volume 50 µl)
5. Add 25 µl of 1% (v/v) chicken RBC's to each well (total volume 75 µl).
6. Mix by gently tapping and leave for 40-60 min at room temperature or till control RBC's settled to a distinct button.
7. HA is easily determined by tilting the plate to observe the presence of tear shaped streaming of the RBC's.
8. HA titre is the one before the tear drop where complete HA (no streaming) occurs.
9. Calculate 4 HA units for HI test.

Haemagglutination Inhibition Test (HI)

1. Column1 of each plate is for controls
    a. RBC control: 50 µl of PBS + 25 µl of RBC's
    b. Virus control: 25µl PBS + 25 µl antigen + 25 µl RBC
2. Add 25 µl of PBS to each well of a U-bottom micro-titre plate in Column 2
3. Add 25 µl of antiserum all wells in Column 2
4. Make two fold dilutions of 25 µl of the virus antigen across the plate, discard final 25 µl
5. Add 25 µl of 4 HA antigen to all wells

6. Incubate at room temperature for 30 min
7. Add 25 µl RBC's to all wells
8. Mix by gently tapping and leave for 40-60 min at room temperature
9. HI is easily determined by tilting the plate to observe the presence of tear shaped streaming of the RBC's
10. The HI titres is the reciprocal of the highest dilution showing complete inhibition and the HI geometric mean titres expressed as reciprocal $\log_2$

Appendix II

(Data collection sheets)

Result sheet for IBV clinical signs

Group.....

Date	Day	dpi	Comments

THE UNIVERSITY
of LIVERPOOL

Multiwall data sheet ELISA or HI

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A												
B												
C												
D												
E												
F												
G												
H												

Result sheet for counting apoptotic cells in TOC rings by TUNEL assay

Group…….Time point ……

Field	Number of Tracheal ring				
	1	**2**	**3**	**4**	**5**
1					
2					
3					
4					
5					

Sheet for histopathological lesions scoring for trachea

Lesion of trachea	**Days post infection**									
	1		**3**		**7**		**9**		**14**	
	Groups									
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Epithelial deciliation										
Epithelial degeneration										
Decrease mucous cells										
Heterophil infiltration										
Epithelial hyperplasia										
Lymphoid infiltration										

Sheet for histopathological lesions scoring for kidney

Lesion of kidney	**Days post infection**									
	1		**3**		**7**		**9**		**14**	
	Groups									
	A	**B**	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Epithelial degeneration										
Ducto-tubular dilation										
Heterophil infiltration										
Lymphoid infiltration										
Epithelial regeneration										
Epithelial hyperplasia										
Lymphoid nodules										

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Result sheet for IHC data

Group……Time point ……

Flied	Number of birds (Tracheal ring)				
	1	**2**	**3**	**4**	**5**
1					
2					
3					
4					
5					

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of LIVERPOOL

Result sheet for ciliostatsis test

Group.....

Ring No	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10
1										
2										
3										
4										
5										
6										
7										
8										
9										
10										

Appendix III

(Published manuscripts)