Studies on the immunopathogenesis, diagnosis and control of infectious bronchitis and avian metapneumoviruses in chicken

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Faez Awad

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INSTITUTE OF INFECTION
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LIVERPOOL

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Abstract

This thesis describes field and experimental investigations on various aspects of the immunopathogenesis, diagnosis and vaccination of infectious bronchitis virus (IBV) and avian metapneumovirus (aMPV) in the chicken.

The immunopathogenesis of an economically important variant IBV (IS/885/00-like) seen in the Middle East and North Africa was examined in one day old specific pathogen-free (SPF) and commercial broiler chicks (Chapter 3). The virus caused respiratory distress, depression and diarrhoea in both SPF and broiler chicks but the severity was milder in the latter. Mild head swelling was observed in one infected broiler chick at 15 days post infection (dpi) and virus with 100% nucleotide level similarity to the inoculum was detected by reverse transcription polymerase chain reaction (RT-PCR) and virus isolation (VI). In the IS/885/00-like infected SPF chicks, cystic oviducts were found in two female chicks. IBV with 99% part S1 sequence similarity to the initial inoculum was isolated from the cystic fluid.

The protection provided by current commercial vaccines against variant IBV IS/885/00-like and IS/1494/00-like was investigated in day old commercial broiler chicks (Chapter 4). Protection was evaluated based on the clinical signs, gross lesions, tracheal ciliary scores and virus detection by RT-PCR. It was found that administering combined live H120 and CR88 vaccines simultaneously at day old, followed by CR88 vaccine at 14 days-old gave more than 80% ciliary protection against both of the Middle East isolates.

Cellular and local immune responses in the trachea following vaccination of day old broiler chicks with different live IBV vaccines were evaluated (Chapter 5). In addition, protection conferred against virulent IBV was also examined. All vaccination programmes were able to induce measurable levels of CD4+, CD8+ and IgA-bearing B cells in the trachea following vaccination when compared to unvaccinated birds. Expression levels of CD4+ and CD8+ cells varied between the vaccinated groups. Vaccines containing $Mass_2$ combined with $793B_2$ produced good protection against challenge with virulent IBV QX compared to vaccines containing Mass ($Mass_1$ or $Mass_2$) alone or $Mass_1$ with D274 or CR88. All vaccination programmes produced more than 80% protection against homologous (M41 and 793B) challenge.

In Chapter 6, IBVs with high nucleotide level similarity to IS/885/00-like and IS/1494/06-like strains were detected by RT-PCR in a broiler flock exhibiting high mortality and respiratory distress in Libya. For the first time, these findings have highlighted the circulation of variant IBVs in the Eastern part of Libya.

Humoral and cellular immune responses and protection studies in SPF chicks that received live Newcastle disease virus (NDV), aMPV and IBV vaccines in single, dual or triple combinations were examined (Chapter 7). Protection against virulent IBV or aMPV was not affected when the vaccines were given either singly or in combination. There were no significant differences in the mean antibody titres of the NDV-vaccinated groups and they remained above the protective titre. The mean titres of antibodies against aMPV were suppressed when aMPV vaccine was given with other live vaccines but the aMPV-vaccinated groups were fully protected when challenged with virulent aMPV. The mean titres of antibodies were similar in the IBV-vaccinated groups and all IBV-vaccinated groups gave almost 100% protection against M41 challenge. Between the vaccinated groups, there were no significant differences in the mean numbers of CD4+, CD8+ and IgA-bearing B cells, reflecting similar levels of tracheal cellular and IgA responses irrespective of single, dual or triple vaccine applications. Despite the aMPV humoral antibody suppression, the efficacy of the live vaccines was not compromised when they were given simultaneously to young SPF chicks.

Comparative studies in day old SPF chicks using both aMPV subtype A or B, separately or in combination, were evaluated (Chapter 8). There were significant differences in the degree of the clinical signs induced by the single subtypes A, B or A+B given together, with most severe signs observed in the latter two groups. By RT-PCR or VI, subtype B virus persisted longer than subtype A. Even though similar titres of the viruses were used, birds given subtype B alone or in combination showed a greater increase in antibody titres than those given A. These findings demonstrate that for the two strains used, subtype B was more pathogenic than subtype A and was excreted and persisted in the tissues for longer.

The use of Flinders Technology Associates (FTA) cards for detection of several avian pathogens has been previously reported. To date, no information has been published on the use of FTA cards for detection of aMPV. In Chapter 9, the feasibility of using FTA cards for the molecular detection of aMPV subtype A and B by RT-PCR was investigated. Findings showed that FTA cards are suitable for collecting and transporting aMPV-positive samples, providing a reliable and hazard-free source of RNA for molecular characterization.

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 July 2011 and September 2014, 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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Faez Awad

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Dedication

This thesis is dedicated to my parents, my wife Nasreen and my children Muhammed, Rhianne, Rawaad and Besaan for their endless love.

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Veterinary poultry research is rarely an individual effort, and so it is with this thesis. At every stage over the last 3 years I have been advised and assisted by many people and without them this project could not have been completed.

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List of publications and presentations

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Conference Publication

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- Ganapathy, K., Awad, F., Forrester, A., Baylis, M., Lemiere, S. and Jones, R. (2013) Interactions between live Newcastle disease, infectious bronchitis and avian metapneumovirus vaccines in specific pathogen-free chicks. *XVIIIth Congress WVPA, Nantes, France*, pp 395.
- Awad, F., Forrester, A., Jones, R., Capua, I., Baylis, M., Chhabra, R. and Ganapathy, K. (2013) Immunopathogenesis of infectious bronchitis virus related to IS/1494/06 and IS/885 in specific pathogen-free chicks. *XVIIIth Congress WVPA, Nantes, France*, pp 385.
- Awad, F., Baylis, M., Jones, R. and Ganapathy, K. (2013) Comparative pathogenesis of avian metapneumovirus subtypes in SPF chicks. *XVIIIth Congress WVPA, Nantes*, France, pp 394.

Presentations at national/ international meetings

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- Awad, F., Forrester, A., Baylis, M., Lemiere, S. and Ganapathy, K. Immune responses and interactions following simultaneous application of live Newcastle disease, infectious bronchitis and avian metapneumovirus vaccines in specific pathogen-free chicks. 8th International symposium on avian corona-and pneumovirus infections/2nd cost action meeting. Rauischholzhausen, Germany, 20th-23rd, June 2014 (Oral presentation).

List of abbreviations

AF	Allantoic fluid
AIV	Avian influenza virus
aMPV	Avian metapneumovirus
ANOVA	Analysis of variance
Ark	Arkansas
ART	Avian rhinotracheitis
FAdV	Fowl adenovirus
bp	Base pair
CD	Ciliostatic dose
CD 4 or 8 (+)	Cluster of differentiation 4 or 8 (positive)
cDNA	Complementary deoxyribonucleic acid
CMI	Cell mediated immunity
Conn	Connecticut
CTL	Cytotoxic T lymphocyte
DNA	Deoxyribonucleic acid
dpc	Days post challenge
dpi	Days post infection
dpv	Days post vaccination
E	Envelope
ECE	Embryonated chicken egg
EID_{50}	50% egg infectious doses
ELISA	Enzyme-linked immunosorbent assay

EXO	Exonuclease
F	Fusion
G	Glycoprotein
HA	Haemagglutination
HEPES	2-hydroxy-ethyl-piperazine-N2-ethane sulphonic acid
HG	Harderian gland
HI	Haemagglutination inhibition
hMPV	Human metapneumovirus
IB	Infectious bronchitis
IBD	Infectious bursal disease
IBV	Infectious bronchitis virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IHC	Immunohistochemistry
ILTV	Infectious laryngotracheitis virus
Kb	kilobases
M	Membrane
M2	Second matrix
Mab	Monoclonal antibodies
Mass	Massachusetts
MDA	Maternally derived antibody
mRNA	Messenger RNA
N	Nucleocapsid
NDV	Newcastle disease virus

ODs	Optical density
OP	Oropharyngeal
P	Phosphoprotein
PBS	Phosphate-buffered saline
qRT-PCR	Quantitative real time reverse transcriptase polymerase chain reaction
RBC	Red blood cell
RFLP	Restriction fragment length polymorphism
RNA	Ribonucleic acid
RT	Room temperature
RT-PCR	Reverse transcriptase polymerase chain reaction
S	Spike
SAP	Shrimp alkaline phosphatase
SEM	Standard error of the mean
SH	Small hydrophobic
SHS	Swollen head syndrome
SW	Sterile water
TBE	Tris-borate-EDTA
$TCID_{50}$	50% tissue culture infectious dose
TOC	Tracheal organ cultures
VI	Virus isolation
VNT	Virus neutralization test

Chapter 1: Literature review

Part I: Infectious bronchitis virus

I.1.1. IBV –a short history

Infectious bronchitis (IB) was first observed in 1931 by Schalk and Hawn in North Dakota in the USA in 2-3 week old chickens (Schalk and Hawn, 1931). However, the nature of the infectious agent was not determined at that time and was assumed that IB was mainly a disease of young chickens. However, it was later observed in laying flocks and the nature of the infectious agent was not determined at that time, which actually may have been a mild form of infectious laryngotracheitis (ILT) (Bushnell and Brandly, 1933). In 1936, it was proved by cross-immunity that the IBV was distinct from ILT (Beach and Schalm, 1936). In 1937, the virus was propagated in the allantoic cavity of embryonating eggs (Beaudette and Hudson., 1937). In 1941, Delaplane and Stuat suggested that IBV propagated in embryonating chicken eggs (ECE) might have immunizing value. The first IBV vaccine in the United States was developed using the van Roekel M41, which is a Massachusetts (Mass) serotype of the virus, and was isolated at the University of Massachusetts in 1941 (Jackwood and de Wit, 2013). In 1956 researchers in Connecticut identified a strain (Connecticut strain) that showed distinct antigenic differences from the Mass strain (Hitchner, 2004). This led to the awareness that different serotypes of the virus exist and that they did not cross protect (Jackwood and de Wit, 2013). In 1962, nephritic disease related to IB in the USA was first reported (Winterfield and Hitchner, 1962). The following year an IB outbreak causing severe kidney lesions in chickens was reported in Australia (Cumming, 1963). In 1956, IB was diagnosed and isolated for the first time in the Netherlands (Bijlenga et al., 2004). This virus was used to prepare a vaccine by serial passage in ECE up to 52nd level passage in early 1960s. The vaccine was named IBV H52 (Bijlenga et al., 2004). Most of the

work in 1970s and 1980s involved identification of different serotypes of the virus by serum neutralization tests. In the 1990s several laboratories began identifying the type of IBV using molecular techniques (Jackwood et al., 1997; Keeler et al., 1998; Worthington et al., 2008). This allowed for the rapid identification of many isolates and the comparison of the viruses around the world. Since then, a wide range of different IBV serotypes and genotypes have been detected globally and the emergence of new and variant IBVs is rapidly increasing (de Wit et al., 2011a; Jackwood, 2012).

I.1.2. The disease

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 most common route of transmission is the airborne one. The spread between flocks where the farms are in close proximity is also likely to occur by aerosol (Jackwood and de Wit, 2013). Other important routes of spread may include contact with faeces and fomites, since the virus has been reported to replicate in the chicken's intestine (Ambali and Jones, 1990). Vertical transmission has been reported (Cook, 1971) but is considered to be of little importance. The morbidity rate can reach 100% but the mortality rate depends on the presence of secondary infection, flock age, immune status, management and environmental factors (Ganapathy, 2009). In young chicks the mortality rate is typically 25-30 % but it can approach 80%, depending on the virulence of the strain. Even though all age group of chicks are susceptible to IBV, young chicks are more susceptible than older ones (Jackwood and de Wit, 2013).

The most notable signs are those affecting the respiratory tract hence the disease was named IB (Jackwood and de Wit, 2013). 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 also affects the reproductive (Crinion et al., 1971; Jones and Jordan, 1972) renal (Meulemans et al., 1987) and gastro-intestinal systems (Ambali and Jones, 1990; Wang et al., 1998; Yu et al., 2001; Ganapathy et al., 2012; Chacón et al., 2014). It has been reported that the virus can cause infertility in male chickens (Boltz et al., 2004; Boltz et al., 2007).

The typical signs of IBV in chickens of less than six weeks of age are depression, huddling under the heat source, gasping, coughing, tracheal râles, nasal discharge, lethargy, watery eyes and mild swollen sinuses in severe conditions (Jackwood and de Wit, 2013).Young chicks may die due to IBV but in uncomplicated infections in chicken more than five weeks of age the disease may not be serious. In any age group, if IBV is mixed with other pathogens more severe diseases can occur (Smith et al., 1985).

Following infection via the respiratory route the virus spreads to other tissues including the reproductive tract. Infection at an early age may result in false layer syndrome in the case of certain IBV strains (Crinion et al., 1971; Muneer et al., 1986), while infection of laying birds produces a range of effects varying from eggshell pigment changes to production drops (Jones and Jordan, 1971). Layers experiencing egg production loss or quality decline may show mild (Muneer et al., 1986) or no respiratory signs (Cook and Huggins, 1986). However, the severity of the egg production decline in quantity and quality may vary, depending on the IBV strains (Dhinakar Raj and Jones, 1997; Jackwood and de Wit, 2013).

The nephritic form of IBV mostly occurs in broiler chickens (Butcher et al., 1989) but it might also affect layers (Selim et al., 2013). Initially the virus cause signs of respiratory distress followed by severe signs of wet droppings which may contain excessive urate, increase in water intake and high mortality due to kidney damage (Cumming, 1963; Meulemans and van den Berg, 1998).

Swollen head syndrome (SHS) is a condition where chickens have swollen heads due to fascial cellulitis and swollen eyelids. Although SHS has been associated with aMPV (Picault et al., 1987; Jones et al., 1991; Aung et al., 2008), a *coronavirus* was isolated by Morley and Thomson (1984) in the first description of SHS in Southern Africa. IBV M41 has also been isolated from commercial broiler flock in the Central Valley of California experienced respiratory disease and signs of SHS (Droual and Woolcock, 1994). The role of IBV in the pathogenesis of SHS is not certain although it has been postulated that *Escherichia coli* (*E.coli*) may invade the air spaces of the cranial bones through the Eustachian tube following an upper respiratory tract viral infection (Droual and Woolcock, 1994).

I.1.3. Infectious bronchitis virus properties

Avian IBV is a single-stranded positive sense, enveloped RNA virus that belongs to the Group 3 of the coronavirus genus with other avian coronaviruses (Cavanagh, 2003). The *Coronaviridae* family is, together with the *Arteriviridae* and *Roniviridae*, within the order *Nidovirales.* The virus is round to pleomorphic in shape, possesses an envelope that is 120 nm in diameter with club-shaped surface projections or spikes (Jackwood and de Wit, 2013). The IBV genome is composed of approximately 27.6 kilobases (kb). Four structural proteins have been recognised spike (S), membrane (M), envelope (E), and nucleoprotein (N). The S protein located at the surface of the virion, consists of two subunits, S1 and S2 with

molecular weights of 92k and 84k respectively. Neutralizing antibodies are elicited by the S1 portion. The E protein is essential for virus particle formation, the M protein is exposed 10% at the outer virus surface; the N protein surrounds the single stranded positive sense RNA genome to form the ribonucleoprotein (Lai and Cavanagh, 1997; Jackwood and de Wit, 2013). IBV has an enormous capacity to change both by spontaneous mutation and by genetic recombination (Jackwood and de Wit, 2013).

The virus first attaches to the appropriate host cell receptor followed by entry into the cell either by fusion with the plasma membrane or endocytosis (Li and Cavanagh, 1992). A replicate complex is also encoded, which carries out the unique discontinuous transcription process resulting in five subgenomic mRNAs (Stern and Kennedy, 1980; Stern et al., 1982; Jackwood and de Wit, 2013). At the molecular level, coronaviruses employ a variety of unusual strategies to accomplish a complex programme of replication and gene expression (Masters, 2006). These uncommon strategies are important factors in the generation of the virus's genetic diversity and continuous evolution. The virus replicates in the cell cytoplasm and within 3-4 hours after infection, new virus particles emerge with maximum output per cell being reached within 12 hours at 37°C (Jackwood and de Wit, 2013).

I.1.4. Incidence and geographical distribution

In the USA, the most commonly isolated type of IBV is Arkansas (Ark) (Jackwood et al., 2005), and the presence of Ark-like isolates indicates that this virus continues to change (Nix et al., 2000). Other commonly detected viruses in the USA are Conn and Mass types (Jackwood et al., 2005). In 1992, the Delaware variant was first reported in Delmarva Peninsula region. The Delaware serotype was found to be antigenically unrelated by virus neutralization test (VNT) to reference IBV serotypes

(M41, Ark DPI, Conn and JMK) from North America (Gelb et al., 1997), but it was found to be closely related to the Dutch variant, D1466 (Lee and Jackwood, 2001). California-type viruses were first isolated in the 1990s and were designated California variant (Moore et al., 1998). In 1999, another related but unique virus designated Cal99 was reported (Schikora et al., 2003; Mondal and Cardona, 2007). The S1 hypervariable region of Cal99 genome was most closely related to Ark serotype viruses (Mondal and Cardona, 2007). From late 1997 to 2000, PA/Wolgemuth/98 and PA/171/99 nephropathogenic strains were identified in Pennsylvania (Ziegler et al., 2002). In 2007 and 2008, two new IBV variants were detected in Georgia and South Carolina broilers with respiratory disease. The viruses were distinct from each other and designated GA07 and GA08 (Jackwood et al., 2010).

In Latin America, in addition to the local strains (BR-I and BR-II), Mass type and viruses similar to the 793B type have been reported, which has not been detected in Brazil (Villarreal et al., 2010). In Mexico, several different genotypes have now been isolated include Conn, Mass and Ark type (Jackwood, 2012). In recent years IBVQ1 type, originally isolated in China has been detected in Chile, Peru, Argentina and Colombia (Sesti et al., 2014).

The Australian 'T' strain was the first IBV strain isolated in Australia in 1962 (Cumming, 1963). Many different IBV variants have been isolated and characterized since then IBV has always evolved independently here from the rest of the world due to its geographical isolation. The Australian isolates have been separated into subgroup 1 (classical strains) and subgroups 2 and 3 (novel strains) based on genetic analysis of S1, 3' ends of the genome and serologic cross-reaction (Ignjatovic et al., 2006). Subgroup 1 viruses include the Australia/N1/62 strain and the vaccine strain

Australia/VicS/62. Subgroup 2 viruses included Australia/ N1/88, Australia /Q3/88, and Australia/V18/91. Subgroup 3 strains are represented by isolate chicken/Australia/N2/04 (Ignjatovic et al., 2006).

Until the late 1970s, it was believed that only IBVs of the Mass serotype were important causes of disease in Europe (Jones, 2010; de Wit et al., 2011a). In 1980s disease outbreaks occurred in the Netherlands in H120 or H52 vaccinated flocks (Davelaar et al., 1984). New strains isolated form these flocks and following differentiation by VN, they were described as Dutch strains; D207, D212, D3128 and D3896 (Davelaar et al., 1984). Many IBV variants were isolated, from other European countries including IBV PL-84084 in France (Picault et al., 1986), B1648 in Belgium (Meulemans et al., 1987), 624/I, Fa 6881/97, AZ 27/98, AZ 20/97, and BS 216/01 in Italy (Capua et al., 1994; Zanella et al., 2003) and Spanish strains of 97/314, 98/313, 00/337 and 00/338 (Dolz et al., 2006).

In the UK, Parsons et al. (1992) reported a new type of IBV. This type, mostly known as 793B (also 4/91 or CR88) was shown to have a nucleotide sequence in the hypervariable regions of the S1 spike gene quite distinct from Mass and Dutch variant viruses (Cavanagh et al., 2005). This virus has a global distribution (Cook et al., 1996a), except USA and Australasia (Jones, 2010).

In 2002 the sequence of a new IBV genotype named Italy 02 (It-02) was submitted to the GenBank. Using VNT it was demonstrated that little antigenic relatedness exists between It-02 and some of the reference IBV serotypes (M41, D274, D1466 and 4/91) which means that It-02 can be classed as a new serotype (Dolz et al., 2006). The origin of It-02 is unknown, but it has been spreading predominantly throughout Europe (Worthington et al., 2008). A pathogenic strain of D1466 that has been detected in some Western European countries for three decades, it has hardly

been reported outside Western Europe, although it is very difficult to achieve a sufficient level of protection against this strain through current vaccines and vaccination strategies (Cook et al., 1999a).

The other important genotype in the region is called QX. The virus was first isolated in China in 1996 and associated with proventriculitis (Wang et al., 1998).

In Europe, a QX-like (D388) strain was first detected in 2003 in The Netherlands in young broiler flock that was suffering from nephritis and respiratory signs (de Wit et al., 2011b) then based on RT-PCR survey the QX-like was detected in 2003 in Germany (Worthington et al., 2008), and since then it has been detected in Poland, Belgium, France and Italy (Beato et al., 2005; Domanska-Blicharz et al., 2006).

In the UK, IBV QX-like was first isolated from kidneys and caecal tonsils of backyard flock (Gough et al., 2008) and from the proventriculus of broiler flocks in (Ganapathy et al., 2012). The virus has been associated with nephritis, respiratory distress in broilers and false layer syndrome in breeders and layers (Jones, 2010; de Wit et al., 2011a).

Another IBV genotype, Q1, which was genetically and serologically distinct from IBV classical strains, was reported in China from 1996 to 1998 (Yu et al., 2001). The virus is associated with respiratory disease and proventriculitis in layer chickens. Recently, IBV Q1 strain has also been identified in Europe (Italy) (Toffan et al., 2011b; Toffan et al., 2013).

In the Middle East, the IBV Mass, 793B, QX and Dutch strains has been reported (Abdel-Moneim et al., 2006; Amin et al., 2012; Boroomand et al., 2012). IBV genotypes with certain regional importance such as IBV IS/855/00 (Meir et al., 2004), and IS/1494/06 were isolated in Israel in 2006 (Meir, R. Personal communication, 2012). Later on these strains were also reported in Egypt (Abdel-

Moneim et al., 2012), Iraq (Mahmood et al., 2011). There are also unpublished reports of IBV Q1 detection in Saudi Arabia (K. Ganapathy, unpublished data).

In Western Asia, respiratory disease outbreaks were investigated in Iraq, Jordan, and Saudi Arabia in early 2011. Five IBV isolates (JOA2, JOA4, Saudi-1, Saudi-2, and Iraqi) were detected by RT-PCR. These five IBV isolates were found to be of the IBV strain CK/CH/LDL/97I, that is known to have originated in China and Taiwan (Ababneh et al., 2012).

In Eastern Asia, many published reports have revealed the diversity of IBVs causing disease in different part of China. But, possibly the most significant IBV variant that has emerged from China is the QX variant reported in association with proventriculitis (See Europe section), which spread throughout Russia (Bochkov et al., 2006) and then appeared in much of Europe (Worthington et al., 2008). IBV Q1 was first isolated in China (Yu et al., 2001), and the virus has been reported in Taiwan (Jackwood, 2012). In Korea, a nephropathogenic strain designated KM91 was detected in 1990s. The KM91 type seems to be a major IBV circulating in Korea (Lee et al., 2004).

In North Africa, an unusual enterotropic variant, known as IBV G strain was isolated in Morocco in the early 1980s (El-Houadfi et al., 1986). Interestingly, S1 sequence data have shown that IBV G and 4/91 are very closely related (Jones et al., 2004). In Tunisia, three isolates were identified by molecular and VNT and designated TN20/00, TN200/01, and TN335/01. These isolate were found closely related to European strains including D274 and 793B (Bourogaa et al., 2009). In Libya, the IBV genotype of IS/885/00-like and IS/1494/06-like were detected by RT-PCR in broiler flocks (Awad et al., 2014).

On the other hand, the major strain of the USA, the Ark strain, has hardly been reported outside the USA. In brief, variants of most importance in major parts of the world, such as 793B or QX that have spread over Asia, Europe and Africa in a short period have not been reported in the USA or Australasia. It seems likely that geographical isolation and control measures employed in countries may play a part in preventing entry of IBV variants (de Wit et al., 2011a).

I.1.5. Host range

It is accepted that chickens are the most significant natural hosts of IBV (Jackwood and de Wit, 2013). However, IBV has also been isolated pheasants in association with a history of clinical respiratory disease, reduced egg production (Spackman and Cameron, 1983; Gough et al., 1996) or nephritis (Gough et al., 1996). There is more evidence which suggests that IBV has a wider host range than was previously thought where it is not only limited to galliform birds (Cavanagh, 2007). Mass type was detected in pigeons (*Columba livia*) (Felippe et al., 2010). However, Group 3 has been detected in geese, duck and pigeon using RNA-sequences (Chen et al., 2013). Most recently IBV-like viruses were detected by real time RT-PCR in wild birds which includes swans, mallards, geese and gulls and most of the detected fragment genes seem to be IBV; the most of the frequent lineages of IBV were M41, 793B and QX (Domanska-Blicharza et al., 2014).

I.1.6. Pathogenesis

I.1.6.1. Trachea

The trachea is the main site of IBV replication, following which a viraemia occurs and the virus get widely disseminated to other tissues. Many studies have shown virus replication in ciliated epithelial and mucus-secreting cells of the trachea (Yagyu and Ohta, 1990; Nakamura et al., 1991; Owen et al., 1991; Benyeda et al., 2010) and the virus has been isolated from trachea (Otsuki et al., 1990; Janse et al., 1994; Lee et al., 2002). Persistence of the virus in the trachea may vary with strain. For examples, isolation of strain G has been reported in the trachea up to 14 dpi (Ambali and Jones, 1990), while IBV 793B was isolated from the trachea of infected SPF chicks for up to 7 dpi (Dhinakar Raj and Jones, 1996a). Some live vaccine strains such as the Ark strains have been shown to persist (28dpi) in the trachea especially when administered in combination with Mass strains (Alvarado et al., 2006).

It has been suggested that the tracheal damage in terms of ciliary activity is variable since is depends on the virulence of IBV strains for the trachea (Otsuki et al., 1990; Dhinakar Raj and Jones, 1997). The severity of the ciliostasis caused by 793B strain proved to be the mild, while the effect of M41 was more severe (Benyeda et al., 2009). IBV strains damage the respiratory epithelium, often predisposing young chickens to secondary infections such as *E. coli*. Experimental infection with virulent M41 followed by administration of *E. coli* produced airsacculitis, pericarditis, and perihepatitis, which often observed under field condition (Peighambari et al., 2000; Matthijs et al., 2005), while administration of IBV alone failed to induce lesions (Peighambari et al., 2000).

I.1.6.2. Kidney

In addition to replicating in trachea, IBV associated with nephritis can also replicate mainly in the lower nephron of the kidney down to the collecting duct epithelial cells. Evidence shows that the virus replicates in all segments of tubules and ducts, but more often in the epithelial cell of the collecting ducts (Cavanagh et al., 1997). Importantly, high titres of the virus in kidney do not correlate with overt kidney disease. For instance, Moroccan G strain replicates to similar titres in the kidney or trachea (Ambali and Jones, 1990).

I.1.6.2. Oviduct

IBV can replicate in the epithelium of the oviducts in young chicks (Crinion and Hofstad, 1972a) and in laying hens (Jones and Jordan, 1971). Pathogenicity of IBV strain for the oviduct varies, as some IBV strains cannot replicate in the oviduct, neither causing lesions nor showing significant amount of viral antigens in the epithelium cells (Crinion and Hofstad, 1972a). Following infection of day-old chicks with IBV M41, virus was isolated between 5 and 11 dpi (Jones and Jordan, 1972) and was also found to replicate in the epithelium of the oviducts (Crinion and Hofstad, 1972a; Crinion and Hofstad, 1972b; Jones and Jordan, 1972).When laying hens were infected with IBV M41, viral antigen was demonstrated in the epithelium of the oviducts between 6 and 9 dpi (Jones and Jordan, 1971).

I.1.6.3. Other tissues

IBV has also been isolated from a variety of other tissues such as lung, airsacs, oesophagus, proventriculus, duodenum, jejunum, liver, spleen, bursa of Fabricius, caecal tonsils, ileum, rectum, cloaca, semen and eggs (Cook, 1971; Ambali and Jones, 1990; Lucio and Fabricant, 1990; Otsuki et al., 1990; Dhinakar Raj and Jones,

1996a) and virus isolation from the Harderian gland (HG) has been reported following experimental infection with live attenuated H120 (Toro et al., 1996).

I.1.7. Gross pathology changes

I.1.7.1. Respiratory tract

Infected chicken have serous or catarrhal exudate in the nasal passages, sinuses and trachea. The incidence of nasal exudate in infected chickens has been used to assess the severity of disease in different lines of chickens (Parsons et al., 1992). Caseous casts may be seen blocking the lower trachea or bronchi which may result in the death of bird by asphyxiation. Small areas of pneumonia may be observed in the lungs. The air sacs may foamy during the acute infection and then became cloudy and contain yellow caseous exudate (Jackwood and de Wit, 2013).

I.1.7.2. Kidney

The kidneys infected with nephropathogenic IBV are swollen and pale with tubules and ureters distended with urates. The kidney weight and kidney asymmetry are increased (Cumming, 1963). Despite lack of gross lesions microscopic changes of nephritis may still be present (Winterfield and Albassam, 1984).

I.1.7.3. Oviduct

Cystic oviducts may be consequences of IBV infection at a very young age (Crinion et al., 1971; Crinion and Hofstad, 1972a; Jones and Jordan, 1972; Benyeda et al., 2009; de Wit et al., 2011b; Ganapathy et al., 2012). Gross changes in the oviduct may vary from the presence of a continuous patent but underdeveloped structure to a blind sac projecting forward from cloacae (Jones and Jordan, 1972). The middle third of the oviduct is the most severely affected with area of localised hyperplasia

seen in between normal patent oviducts. Caudal to the hypoplastic regions macroscopic cysts filled with a clear serous fluid may be seen (Crinion et al., 1971).

I.1.7.4. Other tissues

Infection with IBV 793B strain was initially associated with deep pectoral myopathy as another clinical finding in chickens (Gough et al., 1992). IBV serotype 793B was isolated from a broiler breeder flock which had a bilateral myopathy affecting both deep and superficial pectoral muscles. It is characterised by paleness and swelling of the deep pectoral muscles with the presence of occasional facial haemorrhages (Gough et al., 1992; Dhinakar Raj and Jones, 1996a).

I.1.8. Histopathology

I.1.8. 1. Trachea

The nature of the microscopical changes in the infected chicks caused by IBV is similar, but the severity and persistence of the lesions may differ between the IBV strains (Benyeda et al., 2010). The trachea of the infected chicks appears oedematous with extensive loss of cilia, rounding and sloughing of the epithelial cells following infection (Nakamura et al., 1991). Minor infiltration of heterophils and lymophocytes may occur within 18 hours following infection. Regeneration of the epithelium starts within 48 hours and may be completed by 7-10 dpi. Hyperplasia is followed by massive infiltration of the lamina propria by lymphoid cells and a large number of germinal centres, which may be present after 7 dpi. Increase hetrophils can be observed later with lymphoid nodules and fibroblast proliferation (Riddell, 1987).

I.1.8. 2. Kidney

The kidney lesions of IB are principally those of interstitial nephritis (Riddell, 1987). The virus cause granular degeneration, vacuolation and desquamation of the tubular of the tubular epithelium, and massive infiltration of hetrophils in the interstitial acute stage of the disease. Focal areas of necrosis may be seen as well as indications of attempted regeneration of the tubular epithelium. During recovery, the inflammatory cell population change to lymphocytes and plasma cells. In some cases, degenerative changes may persist and result in severe atrophy of one or all of the divisions of the kidneys. In urolithiasis, the ureters associated with atrophied kidneys are distended with uretes and often contain large calculi composed mainly of urates (Riddell, 1987).

I. 1.8. 3. Oviduct

Experimental IBV infection of the oviduct of adult hens showed decrease height and loss of the epithelial cells, dilution of the tubular gland and infiltration by lymphocytes, plasma and hetrophils especially around the blood vessels. Oedema and fibroplasias of the submucosa of the oviduct have been observed (Crinion et al., 1971). Oviduct cysts were observed following IBV M41 infection and microscopic cysts wall was thin, but had an intact internal layer of ciliated epithelium (Crinion et al., 1971). However, the virus serotypes varied in their ability to produce histopathological changes in the oviduct. Two serotypes, Conn and Iowa 609, failed to produce any pathological change. IBV M41 serotype produced the greatest number of changes, followed by Australian T (Crinion and Hofstad, 1972a). However, if young chicks without maternally derived antibody (MDA) become infected, the damage to the oviducts was found to be more severe and permanent (Crinion et al., 1971).

I.1.9. Immune responses

The immune response to IBV infection can be divided into local, humoral and cellular.

I.1.9.1. Local immunity

The importance of locally produced antibodies in the defence of mucosal surfaces against IBV infections has been well documented (Gomez and Raggi, 1974; Nakamura et al., 1991; Dhinakar Raj and Jones, 1996a; Dhinakar Raj and Jones, 1996c). Trachea being the main target organ of IBV, IgG, IgM and IgA in that organ have been demonstrated (Nakamura et al., 1991). Following infection with virulent, IBV specific IgG and IgA have been detected in tears, tracheal washes, oviduct washes, duodenal and caecal contents using class-specific monoclonal antibodies in enzyme-linked immunosorbent assay (ELISA) (Hawkes et al., 1983; Dhinakar Raj and Jones, 1996c; Ganapathy et al., 2005b; Meir et al., 2012) and in HG using enzyme linked immune-spot forming (ELISPOT) (van Ginkel et al., 2008). IBV specific IgM was also detected in the tracheal, lung and kidney section of infected chickens using immunohistochemistry (IHC) (Dhinakar Raj and Jones, 1996a). Following infection, from 7 to 21 dpi, high level of the IgG, IgM and IgA cells were detected in the trachea (Nakamura et al., 1991). IBV-specific IgA and IgM secreting cells response in the HG increased following IBV infection (van Ginkel et al., 2008). The removal of the HG resulted in a decreased level of protection against IBV infection in day-old vaccinated chicks (Davelaar and Kouwenhoven, 1980). IgG and IgA levels in tears appear a good correlates of protection against IBV or indicative of effective immunisation, induced by attenuated IBV vaccines (Okino et al., 2013). However, the level of IBV-specific IgG, IgM and IgA varies in the different types of chicken lines (Nakamura et al., 1991).

I.1.8.2. Humoral immunity

In the early studies the antibody response to IBV was described in terms of levels of neutralizing antibodies and HI antibodies (Raggi and Lee, 1965; Cowen et al., 1971; Davelaar and Kouwenhoven, 1977; Gough and Alexander, 1979). The development of IBV specific ELISAs and IHC techniques enabled a more detailed analysis of IBV-specific antibodies and their distribution in different chicken tissues (de Wit et al., 2011a).

IgM is the first antibody detected in serum after IBV infection (Gillette, 1974). Its concentration is maintained in blood for a shorter period than other antibody classes following administration of a virulent (Mockett and Cook, 1986) or an attenuated IBV (Martins et al., 1991). Following infection with IBV M41, significant amounts of IgM were detected in serum between 5 to 18 dpi with highest titres at 8 to 10 dpi and then gradually decline until they became undetectable by 21 dpi (Mockett and Cook, 1986). The IgM response in serum correlated with the protection to challenge with IBV M41 in chickens (De Wit et al., 2010b). Thus, IBV specific IgM antibodies in serum can serve as an indication for a recent IBV infection (Mockett and Cook, 1986) or efficacy of IBV vaccinations (De Wit et al., 2010b).

IgG, the major circulating Ig, is the antibody detected by HI and an ELISA developed to measure it is more sensitive (Mockett and Darbyshire, 1981). Anti-IBV IgG can be detected as early as 3dpi, reaches a peak at about 21 days but can remain in high titre in the serum for many weeks (Mockett and Darbyshire, 1981). After reinfection, IgG levels in sera were much higher than those observed in the primary infection response. IgG persisted for a longer period than IgM following IBV infection (Mockett and Cook, 1986). This is the antibody measured in conventional

serological tests to monitor IBV infections or vaccine uptake (Dhinakar Raj and Jones, 1997).

A previous study has reported the differences in the humoral antibody response following vaccination and it related to vaccination dose and application route. Application of different dose of live H120 vaccine resulted in a dose-related IgA response in the lachrymal fluid (Toro et al., 1997).

Higher IgG levels in lacrimal fluid were detected by ELISA following vaccination by the ocular route as compared with vaccination via the drinking water or cloaca. All routes of vaccination tested resulted in an increase of specific IgA in lacrimal fluid following vaccination (Toro et al., 1997).

MDA can provide protection against IBV infection at one day and one week but not two weeks (Mockett et al., 1987). It has been shown that chicks hatched with high MDA had excellent protection against IBV infection at day old of age but not one week (Mondal and Naqi, 2001). However, the presence of MDA at the time of the vaccination or infection can delay and decrease the serological response to vaccination or infection (de Wit, 2000). It has been reported that vaccination of 1-day-old chicks did not result in antibodies detectable by VN test, in contrast to vaccination of 6 to 20 day old hens (Davelaar and Kouwenhoven, 1977).

I.1.9.3. Cellular immunity

Infection of IBV leads to the induction of both humoral and cell mediated immune (CMI) response (Mockett and Cook, 1986; Martins et al., 1991; Collisson et al., 2000). Although humoral immunity to IBV in the systemic immune compartment is important, it does not correlate with protection against IBV (Raggi and Lee, 1965; Darbyshire and Peters, 1985). The CMI response is critical in the recovery from IBV infections (Collisson et al., 2000). CD4+ and CD8+ were shown in section of

trachea, lung and kidney following infection with virulent IBV 793B (Dhinakar Raj and Jones, 1996a). The CD4+ and CD8+ response were detected as early as 3dpi and peaked at 10 dpi and begins to decline at the same time the viral load starts to decline and the IgG humoral response appears (Seo et al., 1997).This indicates that the CMI response may be an early stage response to IBV infections (Seo and Collisson, 1997).

However, varying results have been reported for the CD4+ and CD8+ functions in the immunotherapy. CD8+ cells are critical in controlling many virus infections (Whiteside et al., 1993; Pei et al., 2003). It was demonstrated that adoptive transfer of IBV specific CD8+ memory T cells collected from infected birds between 3 to 6 weeks post infection were able to protect chicks from IBV infection (Pei et al., 2003). The IBV-specific cytotoxic T cells (CTL) collected from spleen within the period where CTL response was declining, did not protect against IBV presumably due to the rise in number of apoptotic cells and at later time points after memory cells had been established, 3 to 6 weeks post infection, those CD8+ T cells were protective against infection (Pei et al., 2003). Adoptively transferred CD4+ cells do not seem to be important in initially resolving IBV infection in the chicken (Seo et al., 2000), but for long term virus control CD4+ and B cells were also required (Reddehase et al., 1988; Thomsen et al., 1996). These observations demonstrate the importance of a better understanding of the CMI response following IBV vaccination in chickens. To date, little information is available regarding the CMI responses following IBV vaccination. Okino et al. (2013) reported expression of various cytokine genes in the trachea after vaccination with different doses of live H120 vaccine. Their result showed that vaccinated birds were protected against M41 infection and the protection depends on the levels of CMI responses.

I.1.10. Diagnosis

I.1.10.1. Isolation of the causative agent

To isolate IBV from infected birds, OP, tracheal or cloacal swabs can be collected especially within the first week of the infection. Samples of trachea, lungs and caecal tonsils can be collected during post mortem examination, which can be of particular value in cases in which more than one week may have elapsed since the start of infection. Samples of the kidney and oviduct should be considered depending on the clinical history of the disease (Jackwood and de Wit, 2013). Samples collected from the swabs or organs can be used for inoculation of 10-day old SPF ECE (Hitchner and White, 1955), TOC (Cook et al., 1976) or primary chicken kidney cell culture (Otsuki et al., 1979). Procedures for the VI in ECE or TOC have been described in detail in Chapter 2.

I.1.10.2. Detection of IBV antigens

I.1.10.2. 1. Agar gel precipitation test

The agar gel precipitation test (AGPT) can be used for the detection of IBV antigen. The test is very cheap, fast and requires few laboratory facilities, but is relatively insensitive (de Wit, 2000). For best results, several antisera at different dilutions should be used, to prevent false negative results caused by imbalance of the antigen (Lohr, 1981). In recent decades, this test is rarely used.

I.1.10.2. 2. Immunofluorescent assay

The immunofluorescent assay (IFA), which requires an ultraviolet microscope, is a relatively cheap and fast technique for detecting IBV antigen in chickens, eggs, TOCs and cell culture. The IFA is group-specific when using group-specific monoclonal antibodies (Mabs). When using type specific Mabs, the IFA can be a

type-specific test (De Wit et al., 1995). When using the IFA directly on the tissue from field material, the sensitivity can vary from equal to or much less than VI. However, the sensitivity of the IFA is usually lower than that of VI, especially in the later stages of the infection (de Wit, 2000). Similar to AGPT, this test is not commonly used other than for research work.

I.1.10.3. Detection of IBV genome

RT-PCR can been used to detect the presence of viral genome. This method can be applied directly to samples obtained from infected chickens, but more often the virus is first propagated in *in vitro* to increase viral load and improve sensitivity of the test (de Wit, 2000; Jackwood and de Wit, 2013). Viral RNA extracted from samples can be subjected to RT-PCR with primer pairs that are specific for whole or part of the genome. Primer pairs are designed from hypervariable regions in the S1 gene that are specific for the different IBV strains. Primers specific for IBV serotypes Mass, Conn, Ark, JMK, DE and California (CA/633/85) (Keeler et al., 1998),793B, D274 and D1466 (Cavanagh et al., 1999) have been reported. Universal oligonucleotide primers corresponding to the S1 gene are available and designed to work with many types of IBV (Adzhar et al., 1996; Worthington et al., 2008).

Detecting the presence of IBV genome using RT-PCR methods requires additional steps such as sequencing the PCR product, subjecting the DNA to restriction enzyme fragment length polymorphism (RFLP) or hybridization using IBV specific probes (Binns et al., 1985; Jackwood et al., 1992; Moore et al., 1998). Another method for detecting IBV genome is through the use of nested RT-PCRs, which is more sensitive than the conventional RT-PCR (Cavanagh et al., 1999). In this approach a second amplification step is added using DNA produced from the first PCR. The primers used in the nested PCR would only amplify part of the original product. This

method is rarely used due to the high sensitivity and the increased possibility of false positive results from contaminations (de Wit, 2000; Jackwood and de Wit, 2013). However, positive RT-PCR result is not sufficient for diagnosis, sequences of the RT-PCR product should compared with the corresponding sequences of the vaccine strains (Jackwood and de Wit, 2013).

I.1.10.4. Strain classification

Using *in vivo* or *in vitro* methods, IBV can be classified into three major groups, i) serotype, ii) genotype and iii) protectotype.

I.1.10.4. 1. Serotype

Conventionally serotyping has been performed by VNT in various biological substrates (See VNT below). Serotyping has also been conducted by HI (Toro et al., 1987). Some laboratories have used MAbs that corresponding to epitopes formed by the S1 protein. Within the S1 region of the spike gene, there are epitopes which defines the different strains of viruses. These epitopes are common only within those groups of viruses and therefore induce type specific antibodies (Niesters et al., 1987; Cavanagh et al., 1988; Moore et al., 1997). In the VNT, the antibody binds to type specific antigens and neutralizes the virus while in HI tests the serum prevents agglutination of chicken red blood cell (RBC). Though these tests are used to detect serotype specific epitopes, cross-reactions may occur between serotypes especially when sera is collected from field samples (Jackwood and de Wit, 2013). VNT and HI are not commonly used for serotyping because of the limited availability of the increasing number of reference sera, corresponding to different serotypes, which required for analysis. In the last two decades, more than serotyping, laboratories are

using RT-PCR to produce DNA copies of IBV gene followed by DNA sequences and genotyping (Jackwood and de Wit, 2013).

I.1.10.4. 2. Genotype

Classifying IBV strains based on genotype has become the method most widely used for IBV typing (Jackwood et al., 1992; Cavanagh et al., 1999; Lee et al., 2000), and it replaced HI and VN serotyping for determining the identity of a field strain (Jackwood and de Wit, 2013). This method involves using RT-PCR techniques to amplify parts of the viral genome, mainly the S1 gene, followed by DNA sequencing or RFLP (Lin et al., 1991). The information obtained from these procedures has been used widely in molecular epidemiology studies (de Wit, 2000). Unfortunately, the correlation between genotype and serotypes in IBV strains has been contradictory; while several authors have been reported high correlation (Kwon et al., 1993; Keeler et al., 1998). A number of reports by others has shown conflicting results (Kusters et al., 1987; Capua et al., 1999). Therefore, the use of genotyping as the only tool is not recommended and conventional testing such as serotyping and *in vivo* studies should be paired with genotyping in order to corroborate the results obtained (de Wit, 2000).

I.1.10.4. 3. Protectotype

Protectotypes provide information about the efficacy of vaccines. Strains inducing protection against each other belong to the same protectotype (de Wit, 2000). However, live H120 vaccine was shown to induce protection against challenge from the Australian T strain (Darbyshire, 1985), even though VNT suggested that they are not serologically related (Darbyshire et al., 1979). Protectotype classification is useful because it reduces the large and growing number of serotypes to a smaller

group of protectotypes so that when considering the types of vaccines to use in the field one protectotype may serve the purpose of several vaccine strains (de Wit, 2000). For determining the protectotype of a strain, a cross-immunization study has to be performed and this is labour intensive, expensive and requires many animals and isolation facilities (de Wit, 2000). Alternatively, cross-immunization test have been done using TOCs (Lohr et al., 1991; Dhinakar Raj and Jones, 1996d) and oviduct organ cultures (OOCs) (Dhinakar Raj and Jones, 1996d) prepared from vaccinated chickens. These cultures were challenged *in vitro* with homologous and heterologous viruses to assess tracheal and oviduct cross-immunity. Cross protection was seen between serologically related and unrelated viruses (Dhinakar Raj and Jones, 1996d). At the tracheal level, it was seen that H120 and M41 belonged to the same protectotype as expected. However, serologically unrelated viruses (D274 and 793B) also belonged to same protectotype (Dhinakar Raj and Jones, 1996d), despite a 21% variation in the amino acid sequences of the S1 gene of these viruses (Adzhar et al., 1995). Protectotype has been also seen between Australian T strain and New Zealand A strains of IBV, which are serologically unrelated (Lohr et al., 1991). However, when the challenge is performed in chickens, the whole immune system is available, whereas this is not the case in the TOCs (de Wit, 2000).

I.1.10.5. Amino acid analysis

The amino acid sequences of viral proteins is a very useful instrument to help locate conserved domains in proteins which might be essential for their structure and function, and for epidemiological studies (de Wit, 2000). Sequencing can be performed on any part of the genome, but is most usually performed on a selected part of the S protein gene (Keeler et al., 1998; Cavanagh et al., 1999), but sometimes on the M gene (Cavanagh and Davis, 1993) or N gene (Williams et al., 1992). Based

on sequence data, a phylogenic tree can be made, revealing the genomic relatedness between different strains.

I.1.10.6. Detection of IBV antibody

I.1.10.6. 1. Virus neutralization test

The VNT is the gold standard test for the detection of IBV serotype-specific antibodies (de Wit, 2000). The test can be performed in various biological substrates including ECE (Davelaar et al., 1984; Gelb et al., 1989; Gelb et al., 1991), cell culture (Hopkins, 1974) and TOCs (Darbyshire et al., 1979; Cook, 1984). However, lack of standardization between the different VNT systems and users makes it difficult to compare results between laboratories (de Wit, 2000).

I.1.10.6. 2. Haemagglutination inhibition test

HI test is considered to be strain-specific (King and Hopkins, 1983; Monreal et al., 1985; de Wit et al., 1997). This may limit the use of the HI test to monitor vaccine response. The HI test with strain M41 as antigen performed poorly when used to detect antibodies following vaccination with H120 compared to using H120 antigen (de Wit et al., 1997). Terrigeno et al. (2008), vaccinated birds with Mass and 793B types and then did the HI against M41, 793B, 624/I, It-02 and QX. The HI response to heterologous antigens was lower, compared to homologous antigens. Procedures of preparation of IBV HA antigens and HI test are described in Chapter 2.

I.1.10.6. 3. Enzyme-linked immunosorbent assay

The ELISA assay is a convenient method for monitoring of both the immune status and virus infection in chicken flocks (de Wit et al., 1997). Several commercial ELISA kits for IBV specific antibodies detection are already available. Antibody

titres obtained by ELISA were considerably higher than those obtained by HI (de Wit et al., 1997) or VN (Marquardt et al., 1981). Two serum samples are required, one at the first sign of infection and one a week or more later; delay of the first sampling can prevent detection of seroconversion (Jackwood and de Wit, 2013). The use of two different commercial ELISA kits is described in Chapter 2.

I.1.11. Vaccination

Vaccination and good management practices help in controlling the spread of viral infections. Both live attenuated vaccines and inactivated vaccines are used in IB immunization programmes in broilers and breeders (Jackwood and de Wit, 2013). Live viruses used as vaccines have been attenuated by successive passage in ECE (Klieve and Cumming, 1988). However, extensive passage is avoided to prevent reduction in immunogenicity. The decrease in immunogenicity adversely affects the protection offered by the vaccine against the challenge. However, the immunity induced by live IBV vaccines depends on the replication of the virus in the tissue of the host (Jackwood and de Wit, 2013). Evidence suggests that some of IBV vaccines (e.g. Connaught and Ark vaccine strains) increase in virulence after back passage in chickens, demonstrating the potential for enhancement of the virulence of such vaccines by a cyclic infection in a flock (Hopkins and Yoder, 1986).

The serotypes variation of IBV is due to high point mutation or recombination (Cavanagh et al., 1986b). IBV mutations and recombination may be associated with some management practices like high density, multi-age flock with different antibody levels, different vaccination programmes and methods of application (Gelb et al., 1991). The mutations are natural mechanisms of the defence of the virus to avoid the defensive mechanism of chicken immune system. These results in emergence of variant strains that may have new neutralizing epitopes that may not

be recognized by the antibodies exerted by the use of current vaccines (Gelb et al., 1991). The emergence of multiple serotypes or genotypes has led to the production of different vaccines or vaccination strategies in different countries, depending on the prevalent serotypes, in order to obtain optimal protection.

The appropriate strategy for control of IBV is to use vaccine strains that are similar to those found in a particular area. In areas where this is not possible or where there were no vaccines available for the prevalent strains, vaccination with different serotypes either sequentially or simultaneously provides better protection against challenges with several heterologous strains than vaccination with a single serotypes (Cook et al., 1999a; Alvarado et al., 2003; Martin et al., 2007; Terregino et al., 2008; de Wit et al., 2011b).

For example, in the USA, Mass types, Conn, JMK, Ark and Cal99 are currently used for vaccination programme (Gelb et al., 1989; Alvarado et al., 2003; Martin et al., 2007). Vaccination using the live Ark strain is widespread within the USA and offers good protection against circulating field strains of Ark type (Sander et al., 1997) but has not eliminated the strain from the poultry population (Nix et al., 2000). When combined with Mass type the vaccine has been shown to offer good cross protection against other strains of IBV including Conn, JMK, M41, Ark DPI and other variant strains (Gelb et al., 1991; Gelb et al., 2005).

In Europe and Asia, the Mass types, 793B (4/91 and CR88) and D274 vaccines are used (Cook et al., 1999a; Terregino et al., 2008; de Wit et al., 2011b). In some counties in Asia, vaccines based on the local strains have been used widely (Lin et al., 2005; Lee et al., 2010).

Combination of two serologically different vaccines (e.g. Ma5 and 4/91) have been shown to give wide immunity against many heterologous strains (Cook et al., 1999a;

Cook et al., 2001a; Terregino et al., 2008), and this vaccination protocol was found more effective than revaccination with a vaccine of the same serotype as the first vaccine (Cook et al., 1999a). Live vaccines combinations of IBV with NDV or aMPV or infectious laryngotracheitis virus (ILTV) have been used in the vaccination programme. However, it has been shown that IBV interference with the replication of attenuated aMPV, although it did not prevent the induction of protective immune responses by the aMPV (Cook et al., 2001b). Quantitative RT-PCR was successfully used to evaluate the replication interference potential of IBV and NDV after vaccination of chickens with live vaccines administered together either in manufactured combinations or single entity preparations (Gelb et al., 2007). Protection induced by combined ILTV and IBV, the results indicate that IBV did not interfere with the protection induced by ILTV vaccine (Vagnozzi et al., 2010).

Experimental administration of live IBV vaccine can be performed individually by oculonasal (Cook et al., 1999a) or intratracheal routes (Yachida et al., 1986). In the field the live vaccines are usually applied by mass application methods include coarse spray aerosol, and drinking water (Jackwood and de Wit, 2013). Mass administration methods are popular because of convenience, but problems in attaining uniform vaccine application can occur, and the aerosol method may cause more severe respiratory reactions. Broiler are most commonly vaccinated with live IB vaccine at one day old in the hatchery and then revaccinated with same or different serotype at 10-18 days of age (Jackwood and de Wit, 2013).

Inactivated oil-emulsion IBV vaccines are commonly used to obtain long-lasting immunity to protect breeders and layers prior to the onset of the egg production (Cavanagh, 2003). Single applications of inactivated virus induce little or no protection against egg loss (Muneer et al., 1986; Cavanagh, 2003), and no protection

against loss of ciliary activity in the trachea (Martins et al., 1991). Significantly higher protection against the infection has been observed when birds are vaccinated at least twice with inactivated IBV(Gelb et al., 1989). The common approach in the poultry industry is to vaccinate young females with live vaccines two or more times, followed by one dose of inactivated vaccine before the birds come into lay. However, the efficacy of inactivated vaccines depends on the proper priming with live vaccines (Cavanagh, 2003; Jackwood and de Wit, 2013). Inactivated vaccines are given individually by intramuscular or subcutaneous injections for induction of high levels of humoral antibodies, which will protect the oviduct of the birds against circulating IBVs and avoids drops in egg production or quality. Such vaccination in breeder birds helps in ensuring transfer of high and uniform levels of MDA to hatching chicks (Jackwood and de Wit, 2013). MDAs are important in conferring some protection against of virulent IBVs (Davelaar and Kouwenhoven, 1977; Mondal and Naqi, 2001).

Part II: Avian metapneumovirus

II.1.1 History

Avian metapneumovirus was first isolated in 1978 from sinus exudates of turkeys in South Africa (Buys et al., 1989). Subsequently, aMPV isolations were reported in the UK in 1985 (Anon, 1985) , Germany (Hafez et al., 2000), France (Picault et al., 1987), Israel (Banet-Noach et al., 2005) Brazil (Chacón et al., 2011). Apart from Australasia, all major poultry rearing regions of the world have reported the presence of aMPV infection (Jones and Rautenschlein, 2013). It is also referred to as turkey rhinotracheitis virus (TRTV) or avian rhinotracheitis virus (ARTV).

II.1.2. Aetiology

II.1.2.1. Classification

Avian metapneumovirus is one of the two viral species, with human metapneumovirus (hMPV), belonging to the Metapneumovirus genus; Metapneumoviruses are part of the subfamily *Pneumovirinae* within the *Paramyxoviridae* family, including single stranded, negative sense RNA, and enveloped viruses (Pringle, 1998; Jones and Rautenschlein, 2013).

II.1.2.2. Morphology

Negative staining electron microscopy, aMPV appears as highly pleomorphic fringed particles ranging from 50 nm to more than 200 nm in diameter. These are frequently spherical, but often appear in long filamentous form, over 1000 nm in length, particularly in preparations from organ culture propagation. Their nucleocapsids have a helical shape and on the envelope surface projections of about 13-14 nm are clearly distinguishable (Cook and Cavanagh, 2002; Jones and Rautenschlein, 2013).

II.1.2.3. Genome

The virus genome is non-segmented and composed of single stranded negative sense RNA of approximately 14 kb (Jones and Rautenschlein, 2013). The virus has the following genes: Nucleocapsid (N), phosphoprotein (P), matrix (M), fusion (F), second matrix (M2), small hydrophobic (SH), surface glycoprotein (G) and viral RNA-dependent RNA polymerase (L), flanked by a leader and trailer at the 3′ and 5′ ends, respectively (Ling et al., 1992; Easton et al., 2004).

II.1.2.4. Virus attachment, entry and replication

The G protein enables virus attachment on the cell receptors, while F promotes the fusion of the envelope with the cell membrane; the nucleocapsid is then released in the cytoplasm, where aMPV replication occurs (Easton et al., 2004). aMPV follows the generally accepted replication and transcription models for other Mononegavirales (Fearns et al., 2002). Negative single stranded RNA viruses require the ribonucleic complex both for transcription and replication. This means their genome needs to be encapsidated with N, P and M2 protein together with the viral polymerase to initiate the infectious cycle (Jones and Rautenschlein, 2013).

II.1.3. Subtypes of aMPV

Avian metapneumovirus is classified into four subtypes based on nucleotide and deduced amino acid sequence data (Pringle, 1998). Subtype A (aMPV/A) and B (aMPV/B) are prevalent in Europe and a number of other countries (Cook, 2000; Juhasz and Easton, 1994). Subtype C (aMPV/C) (Colorado) was isolated in USA (Seal, 1998), France (Toquin et al., 1999), Korea (Lee et al., 2007), and more recently isolated from chickens in China (Wei et al., 2013). A phylogenetic study revealed that subtypes A and B were more closely related to each other than to subtype C (Seal, 1998). Subtype D (aMPV/D) isolated in France showed a low G gene sequence identity with aMPV A , B and C subtypes (Bäyon-Auboyer et al., 2000). Sequence data have revealed that hMPV is more closely related to subtype C than to the other subtypes (Toquin et al., 2003). In addition, when the four subtypes were compared in the phylogenetic analysis, subtype A, B and D were more closely related to each other than to subtype C (Bäyon-Auboyer et al., 2000).

II.1.4. Host range

Natural hosts of aMPV are turkeys and chickens (Cook, 2000). However, guinea fowl and pheasants have been shown to be susceptible to the infection (Gough et al., 1988). Antibodies to aMPV have been detected in farmed ostriches in Zimbabwe, although no virus was isolated (Cadman et al., 1994). aMPV has been detected by RT-PCR and occasionally isolated form nasal turbinates of sparrows, ducks, geese, swallows, gulls and starlings sampled in the North Central Region of the United States and shown to be closely related to subtype C viruses isolated from commercial turkeys (Shin et al., 2000b; Shin et al., 2002a; Bennett et al., 2004; Bennett et al., 2005). There have also been reports of aMPV/C isolated from

pheasants in Korea (Lee et al., 2007), Muscovy ducks (Sun et al., 2014) and chickens in China (Wei et al., 2013). In Brazil, subtypes A and B have been detected in wild birds and pigeons (Felippe et al., 2011).

II.1.5. Virus transmission

Bird-to-bird transmission within flocks seems to occur by direct contact and inhalation of virus-infected respiratory secretions (Cook et al., 1991b; Mcdougall and Cook, 1986). Epidemiological studies indicate that migratory birds may play a critical role in viral transmission in different geographic regions (Cook, 2000; Shin et al., 2000b; Bennett et al., 2004). There is no published evidence that aMPV can be vertically transmitted (Jones and Rautenschlein, 2013), even though high level of virus can be detected in the reproductive tract of laying turkeys (Jones et al., 1988).

II.1.6. Pathogenesis in chickens

Relatively few experimental studies with aMPV had been done in chickens (Jones et al., 1987; Majó et al., 1995; Catelli et al., 1998; Aung et al., 2008). The virus targets the ciliated columnar epithelium of the upper respiratory tract and induces inflammatory lesions in the nasal turbinate, trachea, infraorbital sinuses between 3 to 14 dpi (Majó et al., 1995; Catelli et al., 1998) and can be re-isolated from nasal secretion, nasal turbinate, sinus tissue, trachea to 5 or 14 dpi (Catelli et al., 1998). However, occasionally aMPV can reach the lungs and the air sacs (Cook et al., 1993; Catelli et al., 1998; Van de Zande et al., 1999). Bacterial co-infection such as *E. coli* (Al-Ankari et al., 2001), *Bordetella avium* (Jirjis et al., 2004) and *Mycoplasma gallisepticum* (Naylor et al., 1992) seem to facilitate viral penetration a long lower respiratory tract and to enhance the virus distribution in infected birds. It is still not clearly understood how the virus can infect other organs outside the

respiratory system, but aMPV detected in the reproductive tract (Jones et al., 1988), HG (Khehra and Jones, 1999; Aung et al., 2008) spleen, caecal tonsil and bursa of Fabricious (Aung et al., 2008). No clear differences have been found in the pathogenesis among different subtypes and the different results could be related to the virulence of the single strain rather than to the subtype (Van de Zande et al., 1999; Aung et al., 2008).

II.1.7. The disease in chickens

In chickens the role of aMPV as a primary pathogen was until quite recently questioned and infection may not always be associated with clinical signs (Jones and Rautenschlein, 2013). It is well known that aMPV can infect chickens and induce a specific antibody response (Wyeth et al., 1987) and the chickens have been experimentally infected with aMPV (Jones et al., 1987; Cook et al., 1993; Catelli et al., 1998). However, the disease in turkey is characterized by prominent respiratory signs and in breeder turkey flocks infection causes loss of eggs production (Jones, 1996). In contrast to disease in the turkey, uncomplicated infection in the chicken is frequently so mild that in may go unnoticed unless perhaps SHS develops (Jones, 1996). Decrease in egg production and increase of abnormal eggs due to malformation of egg shell were observed in experimentally infected chicken (Sugiyama et al., 2006). The virus plays a role in the complex disease, SHS, which characterized by apathy and swelling of the face and infraorbital sinuses. Cerebral disorientation, torticollis and opisthotonus frequently follow (O'Brien, 1985; Pattison et al., 1989). The morbidity for SHS is often less than 4 %, but respiratory signs are often widespread in the flock. The mortality is low, usually not more than 2 % (Cook, 2000).

However, aMPV is not the only agent associated with SHS (See above, I.1.2). It had been difficult to produce SHS in chickens under experimental conditions with typical signs of swollen sinuses that had been observed in field outbreak (Jones et al., 1987; Majó et al., 1995; Catelli et al., 1998; Al-Ankari et al., 2001). Aung et al. (2008) reported that aMPV subtypes A and B of turkey origin were pathogenic for broiler chickens, and induced swelling of periorbital sinuses and histopathological lesions in upper respiratory tract.

II.1.8. Immunity

MDAs are passed from hens to their progeny, but their role does not seem to be important as the virus may infect birds with high levels of circulating MDA in turkeys (Naylor et al., 1997a) and chickens (Catelli et al., 1998). aMPV MDA do not interfere with early vaccination, allowing young chicks to be immunized in early stages (Cook et al., 1989b). Chickens develop high aMPV serum antibody levels at about 2 weeks post infection (Picault et al., 1987; Cook et al., 1993). The virus may induce an antibody response in chickens without obvious clinical signs (Gough et al., 1988). The antibody responses seem to be lower in chickens as compared to turkeys after infection with vaccine or virulent aMPV strains (Cook et al., 1993; Cook and Cavanagh, 2002).Vaccinated turkey poults without a detectable antibody response were protected against challenge with virulent aMPV (Jones et al., 1992). In chickens, no antibodies response to aMPV vaccine, were detected in spray or drinking water vaccinated chicks but increasing level in the oculo-oral group were observed. Despite these differences, all vaccinated birds were protected against the challenges with virulent aMPV (Ganapathy et al., 2010). Humoral antibody levels are not associated with protection against infection. Instead, the local and cellular immunities have been shown to play the major role in protection against disease and

virus clearance (Jones et al., 1992; Ganapathy et al., 2005a). Vaccinated turkey poults, which had been B cell depleted by cyclophosphamide treatment did no seroconvert, but were still protected against challenge with virulent aMPV (Jones et al., 1992). Infected broilers chicks with virulent aMPV subtype A and B showed a clear CD4+ T cell accumulation in the HG at 6 dpi and the specific IgA-ELISA and VN-antibody levels in tracheal washes decreased by 10 and 14 dpi, respectively (Rautenschlein et al., 2011).Vaccination of 1 day-old SPF chicks with live attenuated strains stimulates the release of aMPV-specific IgA in the lachrymal fluid after 14 dpv (Ganapathy et al., 2005a). A study has shown that following respiratory exposure to aMPV subtype C, there was an increase of IgM, IgG and IgA cells in the respiratory mucosa (Cha et al., 2007).

II.1.9. Diagnosis

II.1.9.1. Virus isolation

Isolation of aMPV has proven to be extremely difficult because the virus has a fastidious nature and VI attempts from clinical cases often result in contamination with secondary organisms (Worthington et al., 2003). VI from chickens appears to be more difficult than from turkeys and the reason for this is not understood (Jones, 1996). However, the best material source is from nasal secretions or tissue scraped from the sinuses of affected birds obtained within 6 to7 dpi (Gough et al., 1988; Cook and Cavanagh, 2002; Worthington et al., 2003). Difficulty may be found in isolating virus beyond 6 to 7 dpi and this may be only 3 days after the onset of clinical signs (Jones, 1996).TOCs of turkeys and chickens were extensively used to isolate subtypes A and B in Europe (Cook and Cavanagh, 2002). However, the TOC system was not suitable for the isolation of subtype C (Cook et al., 1999b). Instead,

multiple blind passages in chicken embryo fibroblasts (CEF) were successfully used in the primary isolation of aMPV subtype C (Shin et al., 2002b).

TOC are prepared from tracheal rings of chicken (19 -20 day old) or turkey (8-10 day old) embryos (Cook et al., 1976; Mcdougall and Cook, 1986). After inoculation strains of aMPV subtype A and B cause ciliostasis at 6 to 10 dpi. The virus titre peak is reached at 3 to 5 dpi (Cook et al., 1991b). Procedure of aMPV isolation is described in Chapter 2.

II.1.9.2. *In vitro* adaptation of aMPV to cell cultures

Primary avian cell cultures, namely CEF (Goyal et al., 2000), chicken embryo liver cells (CEL) (Baxter-Jones et al., 1989; Williams et al., 1991) and the mammalian VERO cell line (Williams et al., 1991) can readily be used to adapt any aMPV field strain to the cell culture system. A primary isolation of either subtypes of aMPV can also be conducted in cell culture, such as in VERO cells. This method is even less laborious than isolation in the two host systems described above (Toquin et al., 2006; Guionie et al., 2007).

II.1.9.3. Serology

Extensive efforts have been made on the development of serological methods for detection of aMPV-specific antibodies. Although methods like VNT and indirect immunofluorescence tests exist (Baxter-Jones et al., 1989).

Today a number of commercial aMPV specific ELISA kits are available each of which can be used to test both turkey and chicken sera (Cook, 2000). However, the kits greatly vary in subtype specific sensitivity. This was demonstrated by comparing three commercial aMPV specific ELISA kits. All kits revealed 100% specifity but varying sensitivity (Mekkes and de Wit, 1998). Eterradossi et al. (1995) used in-house and commercial ELISA kits as well as VNT for the detection of aMPV-specific antibodies following vaccination and/or challenge with a range of different attenuated and virulent aMPV isolates. This study revealed that the inadequate choice of coating antigen may totally hinder or interfere with the detection of actually existing antibodies following vaccination or challenge infection. It may be beneficial to use homologous antigen for detection of antibodies of suspected subtype (Toquin et al., 1996; Mekkes and de Wit, 1998).

Detection of aMPV-neutralizing antibodies is done by standard neutralization techniques. This technique is meaningful and adaptable to scientific approaches, but more time consuming and expensive than ELISA technique and for this reason less applicable for serological screening in the field. With respect to the subtype-specific application a variety of host systems, such as CEF, CEL, VERO cells or TOC can be used for VNT (O'Loan et al., 1989; Williams et al., 1991; Cook et al., 1993).

II.1.9.4. Virus detection

Initially detection was conducted by electron microscopy (Mcdougall and Cook, 1986), immunoperoxidase (Catelli et al., 1998) immunofluorescence (Jones et al., 1988; Baxter-Jones et al., 1989) or immunogold (O'Loan et al., 1992) techniques. More recently, RT-PCR provided a tool for specific detection of aMPV. This PCR techniques allow the detection of aMPV or the specific detection of subgroups aMPV A to aMPV D (Cavanagh et al., 1999; Bayon-Auboyer et al., 1999; Bäyon-Auboyer et al., 2000; Toquin et al., 2003). In addition to specific detection of aMPV this technique provides amplicons that can be sequenced for further phylogenetic characterization of the isolate. These days the PCR techniques are complemented by subtype-specific real-time RT-PCR assays (Guionie et al., 2007).

II.1.10. Vaccines and vaccination

Both live attenuated and inactivated aMPV vaccines are available commercially for use in turkeys and chickens. Early work on the attenuation of the virus was difficult due to poor attenuation of the virus and problems in reproducing a suitable challenge model in the laboratory (Tiwari et al., 2006). However, there are now a number of reports on the successful attenuation of aMPV strains in a variety of cell cultures and their effective use as vaccines (Cook et al., 1989a; Cook et al., 1989b; Patnayak et al., 2005). The live attenuated vaccines have been shown to stimulate both systemic immunity and local immunity in the respiratory tract (Khehra and Jones, 1999). Live attenuated vaccines are usually administrated by several methods (oculo-oral, drinking water or spray) (Ganapathy et al., 2010). Live attenuated vaccines are used in growing turkeys and broiler chickens and to prime future layers and breeders for injection of inactivated vaccines prior to onset of lay (Cook, 2000). It is most

important that the live-attenuated vaccines are administered very carefully to ensure that all birds receive an adequate dose at the same time (Cook, 2000).

Administration of a live attenuated aMPV vaccine at 1 week of age did not protect turkey hens against respiratory signs, but provided good protection against drop in egg production and quality (Cook et al., 1996b). A combination of live priming followed by a booster with an inactivated vaccine provided excellent protection against both respiratory infection and drop in egg production in laying turkey.

There is excellent cross protection between subtype A and B in turkey (Cook et al., 1995) and chicken (Santos et al., 2012). However, subtype A and B vaccines protected turkeys against challenge with aMPV/ C, whereas, subtype C vaccines do not protect against A and B subtypes in turkey and chickens (Cook et al., 1999b). Simultaneous vaccination of live aMPV and IBV (Cook et al., 2001b), NDV (Ganapathy et al., 2005a) or aMPV, NDV and IBV (Tarpey et al., 2007) have been reported. In these studies there were reduction in the humoral antibody response to aMPV when co-delivered with IBV and/ or NDV. Despite the reduced antibody response to aMPV vaccine, the birds were fully protected against challenge with virulent aMPV.

Chapter 2: General materials and methods

This chapter describes the general materials and methods used throughout the experimental work. Details of specialised procedures used in specific experiments are included in relevant chapters in which they are applied. Methods for preparing all reagents, buffers and media used in these studies are given in Appendix 1.

2.1. Embryonated chicken eggs

ECE were used for virus propagation, preparation of TOC and as a source of day-old SPF chicks. The SPF eggs were obtained from a commercial supplier[1]. The parent flock was free from all major infectious disease agents, including IBV and aMPV.

2.2. Incubation

Incubation was conducted in a commercial incubator[2] at 37°C with humidity controls and automatic hourly turning. Fertile eggs were used at the ages required.

2.3. Experimental chickens

i) SPF chicks

Fertile SPF eggs were set for incubation at 37°C and checked for their fertility on day ten. They were transferred to a tray at the bottom of the incubator in a static condition for hatching at 21 days (Figure 2.1).

[1] Lohman, Cuxhaven, Germany

[2] Victoria, Fort Dodge animal health, USA

Figure 2. 1. SPF eggs incubated at 37°C for 21 days for hatching.

ii) Broiler chicks

Day-old commercial broiler chicks with IBV MDA were obtained from a local commercial hatchery[3].

iii) Housing and management

Chicks were housed in the experimental poultry facility at Leahurst campus (University of Liverpool) equipped with a supply of filtered air under negative pressure. Footbaths with 2% Virkon[4] disinfectant and protective clothing (including overalls, masks, latex gloves and boots) were used at all times when visiting the birds. Heat was supplied from an electrical heater which also served as a red light source when the main lighting was turned off. This form of lighting prevents any cannibalism amongst the birds. The chicks were placed on a wood shaving litter in the isolation pens; feed and water were provided *ad libitum* as shown in Figure 2. 2. The room temperatures (RT) and humidity were recorded daily.

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[3] Frodsham Hatchery, Frodsham, UK

[4] Antec International, Suffolk, UK

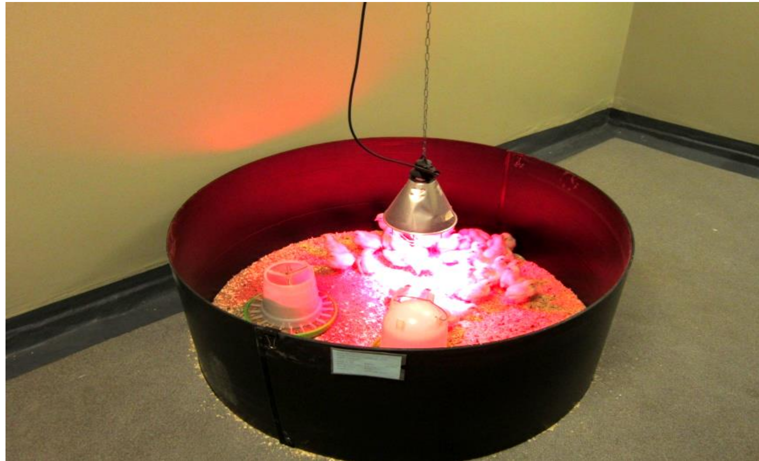

Figure 2.2. Chicks placed on a wood shaving litter in the isolation pens. Feed and water were provided, along with an electrical heater.

iv) Handling of the birds

Stringent precautions were taken when handling the birds in order to reduce the risk of cross contamination between different treatment groups. Disinfectant foot baths were placed both outside and inside each experimental room. Full body disposable gowns were used, and double gowning was operated in rooms when handling infected birds. Disposable gloves were worn and changed in between handling different groups of birds. Disposable face masks were used and also served as a protection for handlers. All used gowns, gloves and face masks were disposed of in disinfectant baths before incineration.

2.4. Source of the viruses and vaccines

Several field and vaccine strains of IBV, aMPV and NDV belonging to different serotype were used in this study; their details are given in respective chapter.

2.5. Examination of chickens

i) Assessment of IBV clinical signs

Chicks were inspected daily to record the clinical signs observed by two persons over a 3 minutes period. Clinical signs were scored as previously described (Grgic et al., 2008). Coughing, head shaking and depression of short during were considered mild signs, whereas gasping, coughing and depression, accompanied by ruffled feathers were scored as severe signs.

ii) Assessment of aMPV clinical signs

Following virulent aMPV challenge (Chapter 8), birds were monitored once a day during the course of infection to record the clinical signs. Chicks were examined individually; their beaks were squeezed gently behind the nostrils to show the presence of nasal exudate (Figure 2.3) and clinical signs were scored as previously described (Jones et al., 1992): no clinical signs = 0, clear nasal exudates = 1, turbid nasal exudates = 2, frothy eyes and/or swollen intraorbital sinuses in conjunction with nasal exudates = 3. Total clinical scores for each group were calculated based on the mean of scores observed per total number of chickens at each dpi.

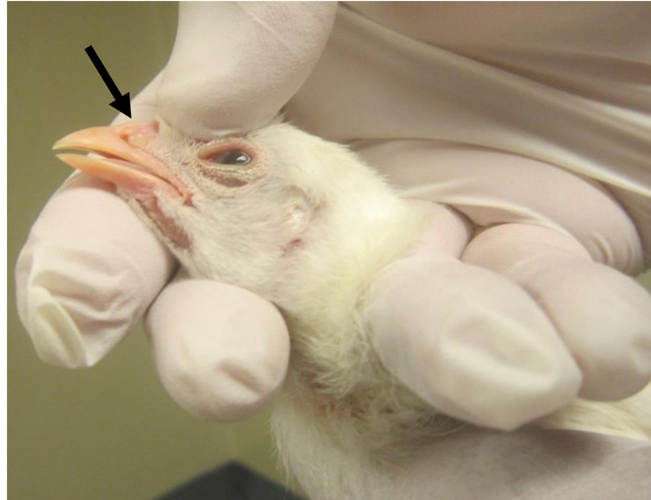

Figure 2.3. Gently squeezing the beak to detect nasal exudates.

iii) Necropsy examination for IBV

Gross lesions were evaluated on all dead or scarified chicks individually and scored according to the following formula described in Table 2.1.

Table 2.1. Shows the scoring system of gross lesions following IBV infection.

Tissue	Lesions	Scores
Trachea	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

iv) Euthanasia of birds

Birds used for tissue sampling, severely affected challenged birds and all birds remaining at the end of each experiment were humanly killed by wing vein injection of 0.5-1 ml of Euthatal[5].

v) Ciliostasis test (IBV)

Following euthanasia, the ciliostasis test was carried out on ten tracheal rings per bird to determine the level of vaccine protection in the trachea. The trachea was removed aseptically and immediately placed in warm (37°C) TOC medium (Appendix 1). Each trachea was cut (3 rings from the upper, 4 rings from the middle and 3 rings from the lower) using a tissue chopper[6] and the trachea was placed in a petri dish[7] containing warm TOC medium and examined under a low power microscope (100x magnification). Cilia beating in each ring was scored as previously described (Cook et al., 1999a): 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% ciliostasis. 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:

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

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

[6] McILWAIN tissue chopper (The Mickle laboratory engineering. Co LTD)

[7] Sterilin Ltd, Hounslow, UK

2.6. Sample collection

i) Blood

Blood samples were obtained from the wing (brachial) vein of each bird using a 23-gauge sterile needle[8] and 2 ml syringe[8] and placed in individually labelled bijou tubes without anticoagulant. The blood was kept in a slant position on a workbench overnight at RT to allow serum separation. Sera was removed the following day and then stored at -20°C until required.

ii) Swabs and tissue

Sterile cotton wool swabs[9] were used throughout all experiments. Following euthanasia, tissues were collected aseptically from each bird and placed in labelled bijou tubes. These samples were securely transported to the laboratory and samples were processed as soon as possible.

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[8] Becton, Dickinson and Co. Ltd

[9] Medical wire and equipment Co. Ltd

2.8. Virological methods

i) Virus propagation and isolation in ECE

Fertile SPF eggs of 9-10 days incubation were used for the propagation and isolation of IBV strains. At the candling eggs were marked around the air space and also at a point on the periphery of the air sac where no blood vessels could be seen on the chorioallantoic membrane. This point was punctured using a pin disinfected with 70% alcohol. The inoculum was introduced to the allantoic cavity by a syringe equipped with a 23-gauge needle. The site of inoculation was sealed with molten paraffin wax and eggs were incubated at 37°C in an incubator. Eggs were examined daily and embryos dying within 24 hours were discarded. End points were decided on the basis of embryo deaths (after 24 hours) and dwarfing of the embryos.

ii) Virus propagation and isolation in TOC

The method for the preparation of TOC has been previously described by (Cook et al., 1976). TOC medium in sterile tubes[10] was used for the cultivation of TOC. The tubes were filled with 0.6 ml of medium and placed overnight in an incubator at 37ºC before the preparation of TOC rings. Embryos were euthanised humanely at 18 to 20 days old, and the entire trachea from the glottis to the syrinx was removed and placed in a petri dish containing warm (37°C) TOC medium. The extraneous tracheal tissue was removed using a small scalpel blade and forceps. The trachea was then placed on sterile filter paper[11] and cut into rings of 0.6 to 0.8 mm thickness using a tissue chopper. Several rings from the upper and the bottom ends were discarded and the remaining rings were placed into the already prepared tubes, with one ring per tube. Tubes were placed in a rotating drum (8 rpm) in a 37ºC

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

[11] Fisher scientific, UK

incubator[12] overnight. The following day, TOC rings were examined microscopically under low magnification (100x) for ciliary activity. Rings with 75-100% ciliary activity were used for virus propagation or titration.

The medium was removed and each ring was inoculated with 0.1 ml of the sample or swab suspension. Three rings were inoculated for each sample. The virus was allowed to be adsorbed at 37°C for one hour, after which 0.6 ml TOC medium was added and tubes were maintained in the roller drum at 37°C. These were examined daily for 7 days for IBV and 10 days for aMPV. Each sample was passaged up to three times in TOC medium and complete ciliostasis on the third passage was taken to be indicative of virus presence. Supernatants were harvested from the three tubes, pooled, aliquoted and stored at -70ºC until required.

iii) Virus titration

Following virus propagation in TOC (for IBV or aMPV) or ECE (for IBV), the infectivity titre of the virus material needed to be determined. Replicates of three tubes of TOC or ECE were inoculated with a tenfold dilution of the virus stock. For TOC, following incubation for up to 7 days (IBV) and for up to 10 days (aMPV) at 37°C, complete ciliostasis was taken as the endpoint. For the ECE, embryo mortality or abnormalities were assessed five days post inoculation. For both TOC and ECE, titres were calculated according to the method established by (Reed and Muench, 1938) and expressed as the median ciliostasis dose (log_{10} CD_{50}/ml) or Egg infective dose (EID_{50}/ml).

[12] Laboratory thermal equipment Ltd. Greenfield, NR Oldham

2.9. Sample processing

i) Swabs for VI

Virus isolation was attempted from either OP or CL swabs. After sampling, the cotton tips were dipped into 1 ml of TOC medium containing 10x the usual concentration of antibiotics (Appendix 1), shaken vigorously and stored at -70°C until required.

ii) Swabs for RT-PCR

Each swab was dipped into a labelled bijou containing 1.5 ml of guanidine thiocyanate (solution D, Appendix 1), then quickly returned to its sleeve. The first swab was dipped back into the guanidine thiocyanate and rotated 5 times. Excess guanidine thiocyanate was removed by rotating and pressing on the side of the tube. Fluid of swabs were then stored at -20°C until required.

iii) Tissue samples for VI and RT-PCR

Tissues were broken up by grinding in a sterile pestle and mortar using a small amount of sterile sand with 1 ml of TOC medium. This was freeze-thawed three times and then stored at -70°C until required. All tissue dilutions were centrifuged at 3,000 g for 10 minutes before being used.

2.10. RT-PCR

i) Extraction of RNA using phenol chloroform method

RNA was extracted using the guanidine thiocyanate-phenol chloroform method as described previously (Chomczynski and Sacchi, 2006). Three hundred microlitres of mixtures of swabs or processed tissue samples were treated with 300 µl of solution D and frozen at -20°C overnight. The mixture was then thawed and transferred into a screw capped Eppendorf tube[13] to which 50 µl sodium acetate[14] and 650 µl phenol chloroform 5:1[14] were added. Tubes were inverted 12 times to mix and then centrifuged at 13,000 g for 5 minutes. The top layer was removed and mixed with 500 µl isopropanol[14] and then precipitated overnight at -20°C. Following this, the sample was immediately centrifuged at 13,000 g for 15 minutes. The supernatant was removed and the precipitate was carefully washed twice with 100% ethanol and then left to air dry. The precipitate was resuspended in 30 µl of resuspension (RS) water (Appendix 1) and left to stand at 4°C for at least 15 minutes. The samples were flick mixed and then quickly spun for 10 seconds before proceeding.

ii) Extraction of RNA using a commercial kit

Total viral RNA was extracted using the QIAamp viral RNA mini kit[15] following manufacturer's instructions. In brief, 140 µl of each virus sample was mixed with 560 µl of lysis buffer, vortexed briefly, incubated for 10 minutes at RT, followed by the addition of 560 µl of 100% ethanol. Following this, the liquid was applied to a spin-column, followed by two steps washings with washing buffers (AW-1 and AW-2) (Appendix 1 for detailed protocol). The RNA was eluted in 60 µl RNase-free water, and used immediately or stored at -20°C for later use.

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[13] Elkay laboratory products Ltd UK

[14] Sigma-Aldrich, UK

[15] QIAGEN, UK

iii) RT-PCR protocol

The RT-PCR method used in this study was performed as previously described for IBV (Worthington et al., 2008), aMPV (Cavanagh et al., 1999) and NDV(Aldous and Alexander, 2001).

Table 2.2. RT- PCR oligonucleotides.

Virus		Oligo	Sequence (5' to 3')	Gene	Product size (bp)	Position in sequences
IBV	PCR 1	SX1+	CACCTAGAGGTTTG T/C T A/T GCAT	S1	393	677-698
		SX2-	TCCACCTCTATAAACACC C/T TT			1148 -1168
	Nested PCR 2	SX3+	TAATACTGG C/T AATTTTTCAGA			705 - 725
		SX4-	AATACAGATTGCTTACAACCACC			1075-1097
aMPV	PCR 1	G6-	CTGACAAATTGGTCCTGATT	G		422-441
		G1+	GGGACAAGTATC T/C C/A T/G AT			1- 17
	Nested PCR 2	G5-	CAAAGAAGCCAATAAGCCCA			401 to 419
		G8+A	CACTCACTGTTAGCGTCATA		268	152-171
		G9+B	TAGTCCTCAAGCAAGTCCTC		361	68 to 87
NDV	Single step PCR	MSF-1R	GACCGCTGACCACGAGGTTA	F	182	-
		MSF-2F	AGTCGGAGGATGTTGGCAGC			-

a) Reverse transcription (RT) reaction

The PCR reaction mixture was prepared at a separate workstation and gloves were worn at all times to prevent contamination.

The RT reaction mixture included superscript II RT and one of the outer (negative) oligonucleotides (Appendix 1). The mixtures were then taken to a separate workstation where 5 µl of the mixture was placed into a 0.5 ml pre-labelled clip top Eppendorf tube[16], after which 2 drops of mineral oil[17] were added. To this, 0.5 µl of RNA was placed under the oil. Positive and negative controls were included in each run. This was thoroughly mixed by vortexing and then centrifuged for 10 seconds at 5000 rpm. The tubes were placed in a thermocycler [18] and run under the following conditions: 42°C for 1 hour, 72°C for 10 minutes and then held indefinitely at 8°C. Immediately after the RT mixture, the following was performed:

b) Nested PCR 1

The total volume mixture for PCR 1 reaction was 20 µl (Appendix 1). Sufficient PCR reaction mixture was made for all the samples including a positive and negative control for a particular run in a 1.5 ml clip top Eppendorf tube.[19] The PCR reaction mixture was thoroughly vortexed before dispensing 20 µl aliquots beneath the oil layer in each tube containing the RT-PCR product. The tubes were placed in a thermocycler and run under the following conditions: 94°C for 15 seconds followed by 35 cycles of 94°C for 10 seconds, 50°C for 20 seconds, and 72°C for 40 seconds and then held indefinitely at 8°C.

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

[17] Sigma-Aldrich, UK

[18] GeneAmp PCR system 9700

c) Nested PCR 2

The total volume of the reaction mixture for one PCR 2 reaction was 24.5 µl (Appendix 1). Sufficient PCR reaction mixture was made for all the samples including a positive and negative control for a particular run in a 1.5 ml clip top Eppendorf tube. This The PCR mixture was thoroughly vortexed before dispensing 24.5 µl aliquots into labelled 0.5 ml clip top Eppendorf tubes, and overlaid with two drops of mineral oil. Then 0.5 µl of the PCR 1 product was added beneath the oil layer, tubes were flick-mixed, quickly spun and finally placed in a thermocycler and run under the following conditions: 94°C for 15 seconds followed by 35 cycles of 94°C for 10 seconds, 50°C for 20 seconds, and 72°C for 40 seconds and held indefinitely at 8°C.

d) Gel electrophoresis

Agarose gels (1.5%) were prepared by dissolving agarose in 1x TBE buffer (Tris-borate-EDTA) (Appendix 1). Once dissolved the agarose solution was cooled and stained with 2 µl nucleic acid solution (Redsafe™).[20] The solution was then poured into a gel mould (12cm x 9cm) Hybaid Electro-4 gel tank .[21]

For each sample, 10 µl of reaction mix was added to 4 µl of loading buffer. After mixing, the 14 µl was added to each of the wells in the gel. A 100 bp ladder (Molecular marker) [22] was included for amplicon analysis. Gels were run at 75V for 55 minutes in 1x TBE buffer. The gels were analysed using UV transillumination.

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[20] iNtRON Biotechnology, Inc

[21] Hybaid Ltd, Middlesex, UK

[22] Amersham Pharmacia Biotech, UK

2.11. DNA sequencing IBV

For sequencing, positive nested PCR amplicons (393 bp) were purified with 0.15 µl Exonuclease 1 (EXO) [23] and 0.99 µl shrimp alkaline phosphatase (SAP) [23] at 37°C for 30 minutes and then at 80°C for 10 minutes to remove any extraneous nucleotides. Purified products along with forward primer (SX3+) were submitted to external commercial laboratory [24] for sequencing.

i) Phylogenetic analysis and nucleotide comparison

Multiple sequence alignments were prepared with Clustal W (Thompson et al., 1994), and phylogenic trees were constructed using MEGA 6 (Tamura et al., 2013) using the Neighbor-joining method with 1000 bootstrap replicates to assign confidence levels to branches. The IBV sequences were aligned and compared with IBV references available in GenBank (National Centre of Biotechnology Information; http://www.ncbi.nlm.nih.gov/) and BLAST searches were conducted to confirm sample identification.

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[23] 78250, Affymetrix

[24] Source bioscience sequencing, Nottingham, UK

2.12. Serological tests

i) ELISA

Serum samples were analysed using BioChek [25] or IDEXX [26] commercial ELISA kits.

a) ELISA BioChek protocol

All steps were carried out at RT. The ELISA plates were adapted to RT for 30 minutes before use. In brief, serum samples were heat treated at 56°C for 30 minutes and each sample was diluted 1:500 by adding 1 µl of sample to 0.5 ml of sample diluent and mixed well by vortexing. After the addition of 100 µl positive and negative control samples to the indicated wells of the ELISA plate, 100 µl of diluted sample were added to the appropriate well of the plate. The plate was covered with a plastic cover and incubated at RT (20-25°C) for 30 minutes for IBV and 60 minutes for aMPV. After incubation, the contents of the plate were aspirated and the plate was washed four times with 300 µl/well of wash buffer. After addition of 100-µl/well of conjugate reagent (Anti-chicken IgG labelled with the enzyme alkaline phosphatase), the plate was covered again and incubated at RT for 30 minutes for IBV and 60 minutes for aMPV. Following four washing steps with 300 µl/well of wash buffer to remove unreacted conjugate, 100 µl/well of substrate reagent in the form of PNPP chromogen was added and the plate was incubated at RT for 15 minutes for IBV and 30 minutes for aMPV. A yellow colour was developed if anti-IBV or aMPV antibodies were present and the intensity was directly related to the amount of antibody present in the sample. The reaction was halted with stop solution (100 µl/well) (sodium hydroxide in diethanolamine buffer).

[25] Gouda, The Netherlands

[26] Hoofddorp, The Netherlands

Optical density (ODs) was determined by measuring absorbance at 405 nm with a microplate reader.[27] Based on the ODs, the sample to positive (S/P) ratios were calculated and used to express the mean (S/P) ratio per group. For the results to be valid the mean of the negative control should be less than 0.30 and the difference between the positive control mean and negative control mean should be greater than 0.15, S/P ratio values greater than 0.2 were considered as positive.

b) ELISA IDEXX protocol

The ELISAs were performed according to the manufacturer's instructions. Briefly, serum samples were diluted 1:500 with sample diluent provided by the manufacturers, and 100 µl of the diluted samples were dispensed into the antigen coated test plates. Samples were incubated for 30 minutes at 25°C and washed 3-5 times with approximately 350 µl of distilled water, per well. Following this, 100 µl of conjugate was added to each well and plates were incubated for 30 minutes at 25°C. Each well was washed again 3-5 times, as described above, and 100 µl of substrate solution was added to each well, and plates were incubated at 25°C in the dark for 15 minutes. A blue colour developed if anti IBV or aMPV antibodies were present. The reactions were halted by adding 100 µl stop solution per well. Sample absorbance was measured at 650 nm with a microplate reader. For both kits, 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. Samples with S/P ratio value greater than 0.2 were considered positive.

[27] Thermo Scientific, Multiskan FC, Finland

ii) IBV HA antigen preparation and HI test

a) IBV antigen preparation

Reference antigens for IBV serotypes M41 and 793B (4/91) were obtained from a commercial source.[28] Previously described methods (King and Hopkins, 1983; Alexander and Chettle, 1977) were used for preparing IS/885/00-like and IS/1494/06-like HA antigens. Ten-day ECEs were inoculated via the allantoic cavity with a 0.1 ml volume of the respective virus stock (10^6 EID_{50}/ml). Following 72-hours incubation at 37°C, the inoculated eggs were chilled overnight at 4°C. The following day, allantoic fluid (AF) was harvested, pooled and then clarified by centrifugation at 1000 g for 30 minutes. The titre of the virus was determined by using TOC. The virus was concentrated from the supernatant by ultracentrifugation[29] at 30,000 g (Rotor SW 41 Ti) for 90 minutes. All centrifugations were carried out at 4°C. The virus pellet was resuspended to 1/100 original AF volume in 2-hydroxy-ethyl-piperazine-N2-ethane sulphonic acid (HEPES) buffer (Appendix 1). The concentrated virus was treated with enzyme phospholipase C, Type 1, from *Clostridium perfringens*[30] by mixing equal parts of enzyme (1 unit/ml) and virus concentrate, and incubating the virus/enzyme mixture at 37°C for 2 hours. The virus/enzyme mixture was tested for HA activity and stored at 4°C for the same day use

[28] Animal Health Service, Deventer, The Netherlands

[29] Beckman Coulter, USA

[30] Sigma-Aldrich, UK

b) Chicken red blood cells

A total of 2 ml of blood was collected from SPF chicks via the wing vein using a sterile syringe, and added to the same volume of Alsever's solution.[31] This was then gently mixed and centrifuged at 1200 g for 5 minutes. The plasma layer was removed by pipetting. After washing three times with phosphate buffered saline (PBS) (Appendix 1) a 1% RBC suspension was prepared in PBS for HA and subsequent HI testing.

c) HA and HI test procedure

HI tests were carried out in microtiter "U" shaped plates.[32] Twenty five µl of antigen suspension was serially diluted in 25 µl PBS (pH 7.5) and an equal amount of 1% RBC suspension was added to each well. All procedures were performed at RT. The test plates were incubated for 45 minutes before the results were recorded. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming). The reproducibility of the HA titre for each IBV type was examined twice. End point dilutions for each virus were as follows: M41 (1:1024), 793B (1:512), IS/885/00-like (1:32) and IS/1494/06-like (1:8).

HI was used to assess antibody level in samples (Chapter 3 and 4). The test was carried out as described by Alexander and Chettle (1977). Prior to conducting the HI test, serum samples were thawed and placed in a water bath at 56°C for 30 minutes to destroy heat labile non-specific agglutinins. Two fold serial dilutions of 25 µl serum were made with PBS in U-bottomed microtiter plates up to the 10th well. Twenty five µl of 4 HA units (U) of IBV antigen were added up to the 11th well.

[31] Sigma-Aldrich, UK

[32] Thermo Fisher Scientific, Denmark

Plates were kept at RT for 30 minutes to allow antigen antibody reaction. After this, 25 µl of 1% RBC suspension was added to each well. The 11th well contained antigen and RBCs as the positive control, with the 12th well containing only RBCs as the negative control. After gentle mixing for up to 10 seconds, RBCs were allowed to settle at RT for 45 minutes and agglutination was assessed by tilting the plates. The HI titre is the highest dilution of serum causing complete inhibition of 4 HAU of antigen. The agglutination is assessed by tilting the plates. Only those wells in which the RBCs stream at the same rate as the control wells (containing 0.025 ml RBCs and 0.05 ml PBS only) should be considered as showing inhibition. Prior to the HI test, back-titration of the antigen was carried out to ascertain the 4 HAU. The HI was performed using positive control antisera against the M41, 793B, IS/885/00-like and IS1494/06-like antigens.

2.13. Immunohistochemistry

i) Tissue sections/cryostat

At post-mortem the trachea (approximately 1cm/length) was collected from each bird, immediately placed in aluminium foil cups containing cryo embedding compound (OCT) [33] and frozen in liquid nitrogen (-190°C). When needed, each sample was retrieved in a self-sealing polyether bag and stored at -70°C until used for sectioning. Frozen tissue blocks were sectioned at 5µm using a pre-cooled cryostat,[34] mounted on glass slides[35] and then kept overnight away from

[33] Solmedia laboratory, Shrewsbury, UK

[34] LEICA CM 1900, Germany

[35] Thermo Scientific, UK

direct light. These were then fixed in pre-cooled (-20ºC) acetone [36] for 10 minutes, air dried at RT for 10 minutes and then stored at -70ºC until stained.

ii) IHC staining

Monoclonal antibodies against chickens IgA were used as a marker for B cells and CD4+ and CD8+ [37] specific monoclonals were used to identify T-helper and T-cytotoxic cells respectively. Staining was carried out as described by Rautenschlein et al. (2011), with some modification. Briefly, slides were incubated for 20 minutes in 0.03% hydrogen peroxide (H_2O_2) diluted in PBS, washed three times in PBS for 2 minutes and incubated with horse serum for 20 minutes. Tissue sections were incubated with primary antibodies [mouse anti CD4+ chickens at 0.5 µg/ml; 1:1000 (clone CT-4), CD8 alpha+ at 0. 25 µg/ml, 1:2000 (clone CT-8), IgA (clone A-1) at 0.5 µg/ml, 1:1000]. Slides were then incubated overnight at 4ºC. After monoclonal antibody incubation, sections were washed in PBS for 2 minutes and incubated with the secondary antibody (goat anti-mouse IgG) for 30 minutes. After washing three times with PBS, sections were incubated with 1% Avidin DH and 1% biotinylated horseradish peroxidase H reagents (ABC reagent).[38] After a further three washes with PBS, colour development was achieved by addition of 4% 3,3-diaminobenzidine (DAB) [38] substrate containing 2% hydrogen peroxide and 2% buffer stock solution. Slides were then washed in super quality water, and tissue sections were counter stained with hematoxylin for 1 minute, followed by washing in tap water for 5 minutes. Finally, sections were dehydrated for 1 minute in 96% ethanol, 2 minutes in 100% ethanol, 3 minutes in 100% ethanol, 2 minutes in 100% xylene, 3 minutes in 100% xylene, 3 minutes 100% xylene and later mounted with

[36] Fisher Scientific, UK

[37] Southern Biotech, Birmingham, USA

[38] VECTOR Laboratories, Burlingame, USA

aquatic mounting medium and covered with a coverslip. All antibodies used for immunohistochemical studies were diluted in PBS and all incubation steps were carried out at RT. The immunostained sections were evaluated using Nikon ECLIPSE 80i microscope.[39] For each sample and each cell surface determinant (CD4+, CD8+ and IgA), immunostained cells were counted in 5 selected microscopic fields at a 400x magnification (fields/trachea). The average number of positive cells per 400x microscopic field was calculated for each sample. Figure 4 shows the immunostaining of the tracheal tissue of birds infected with virulent IBV QX that used for of the standardisation immunohistochemistry assays

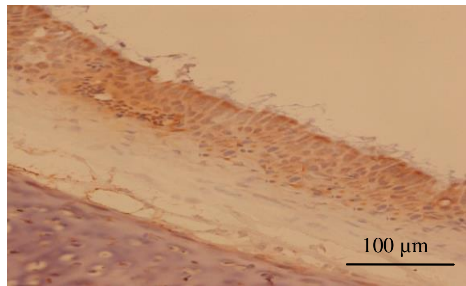

Figure 2.4a. Microscopic image tracheal tissue of control birds

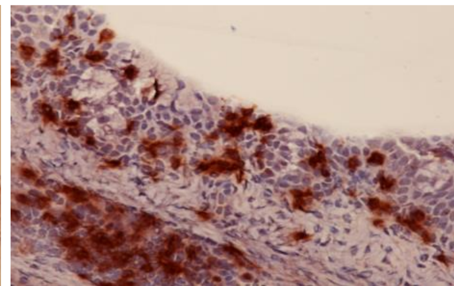

Figure 2.4b. Microscopic image of CD4+ cells in vaccinated tracheal tissue

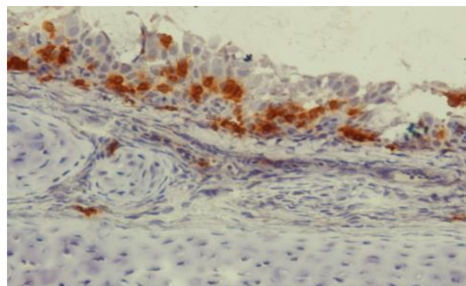

Figure 2.4c. Microscopic image of CD4 cells in vaccinated tracheal tissue

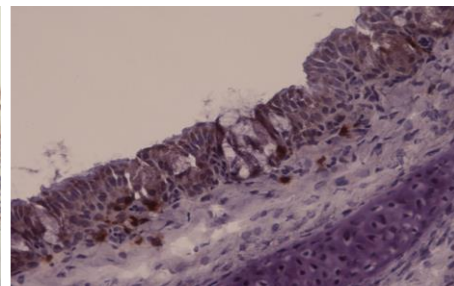

Figure 2.4d. Microscopic image of IgA cells in vaccinated tracheal tissue

[39] Nikon-corporation, Japan

Chapter 3: Pathogenesis of IS/885/00-like infectious bronchitis virus in specific pathogen-free and commercial broiler chicks

3.1 Abstract

The pathogenesis of an IS/885/00-like (IS/885) strain of IBV isolates was examined in one day old SPF and commercial broiler chicks. Following infection at one day-old, chicks were monitored for clinical signs and antibody responses by ELISA and HI. In addition, five chicks were humanely killed at 3, 6, 9, 12, 15, 21 and 28 dpi, lesions were scored, and tissues were collected for histopathology and virus detection by RT-PCR. The IS/885 strain caused respiratory distress, depression and diarrhoea in both SPF and broiler chicks. Mild head swelling was observed in one infected broiler chick at 15 dpi and a virus with 100% nucleotide level similarity to the IS/885 inoculum was detected by RT-PCR and VI. Tracheal and kidney lesions were observed in both types of bird but lesions were more severe in SPF than the broiler chicks. In the IS/885-infected SPF chicks, cystic oviducts were found in two female chicks at 28dpi. IBV with 99% nucleotide level similarity to the initial inoculum was isolated from the cystic fluid aspirated from these oviducts. The SPF showed a higher severity of microscopic lesions and they persisted for longer than in the broiler chicks. The virus was consistently detected in the tissue and swabs throughout the observation period. Increase in antibody titres were found at 21 dpi in both types of birds. The IS/885 strain examined in this study was pathogenic for both SPF and broiler chicks, however the onset of disease was delayed and of lesser severity in the latter.

3.2. Introduction

In the winter of 2000, a severe outbreak of renal disease occurred in several broiler farms in Israel. An IBV, designated IS/885/00, was isolated from the kidneys of these flocks (Meir et al., 2004).

In the Middle East and North Africa, in addition to the circulation of Massachusetts, 793B, QX and Dutch strains (Abdel-Moneim et al., 2006; Bourogaa et al., 2009; Amin et al., 2012; Boroomand et al., 2012), genetically similar strains to IS/885/00 have been reported in Egypt (Abdel-Moneim et al., 2012), Iraq (Mahmood et al., 2011) and Libya (Awad et al., 2014). These strains were isolated from broiler and layer flocks experiencing respiratory distress, renal lesions and high mortality (Selim et al., 2013; Awad et al., 2014). In addition, the IBV IS/885/00 and IS/885/00-like isolates were distantly related to other genotypes reported in both this region and elsewhere (Meir et al., 2004; Abdel-Moneim et al., 2012; Awad et al., 2014).

The pathogenesis and host immune responses of common IBV variants are known, such as M41 (Crinion and Hofstad, 1972a; Jones and Jordan, 1972; Butcher et al., 1990), Beaudette (Geilhausen et al., 1973), Australian T-strain (Chong and Apostolov, 1982; Ignjatovic et al., 2002), Moroccan G strain (El-Houadfi et al., 1986; Ambali and Jones, 1990), 793B (Dhinakar Raj and Jones, 1996a; Boroomand et al., 2012), QX (Terregino et al., 2008; Ganapathy et al., 2012), It-02 (Dolz et al., 2012), Q1 (Toffan et al., 2013) and more recently Brazilian IBV variant (USP-10 and USP-50) (Chacón et al., 2014).

Despite the IS/885/00 strain being one of the predominant IBV strains in the Middle East and North Africa, no published information is available on immunopathogenesis of this increasingly important IBV variant. Furthermore, tissue

tropism and pathogenesis of this novel genotype remain unknown. To better understand the IS/885 strain, an experiment was performed to investigate the pathogenesis in SPF and commercial broiler chicks. Clinical signs, gross and microscopic lesions, virus detection and humoral antibody responses were evaluated.

3.3. Materials and methods

3.3. 1. Virus

The virus used throughout this study (referred here as IBV IS/885) was received as third passage AF from the Istituto Zooprofilattico Sperimentale delle Venezie, Padova, Italy. The virus had been isolated from a recent outbreak of high mortality and respiratory disease complex in broiler flocks in Egypt. Initial isolation was carried out in the virology laboratory at Cairo University, Egypt, and the AF was submitted to the Italian laboratory. There, the AF went through three further passages in ECEs and was shown to be negative for avian influenza virus (AIV) and NDV and positive for IBV by RT-PCR. DNA sequences of the S1 gene showed 99% nucleotide level similarity to the Israeli strain IS/885/00 (Meir et al., 2004).

At the University of Liverpool, the virus received a further passage in 9 to 11 day-old SPF ECE (Chapter 2.1). The viral titre was determined by titration in ECE as $10^{4.66}$ EID_{50}/ml. The AF was free of AIV, NDV, aMPV, ILTV, infectious bursal disease virus (IBDV) and fowl adenovirus (FAdV) by RT-PCR, and free of bacterial or fungal contaminations.

3.3. 2. Eggs and chicks

Fertile eggs from SPF White Leghorn chickens were used (Chapter 2.3.i). Day-old commercial broiler chicks with IBV MDA were obtained from a commercial hatchery (Chapter 2.3.ii).

3.4. Experimental design

3.4. 1. Experiment 1. Infection of SPF chicks

Seventy one-day-old SPF chicks were randomly divided into two groups, consisting of 45 and 25 chicks in the infected and control group respectively. The chicks in the infected group were inoculated oculonasally with 0.1 ml of the virus and those in the control group with virus-free AF.

3.4. 2. Experiment 2. Infection of commercial broiler chicks

Seventy one-day-old commercial broiler chicks were divided into two groups and inoculated as per Experiment 1 described above.

For both experiments, clinical signs were observed daily throughout the experimental period (Chapter 2.5.i). At 3, 6, 9, 12, 15, 21 and 28 dpi, five infected and three control chicks were randomly selected and euthanised to evaluate the gross lesions. OP and CL swabs and tissue samples of trachea, lung, caecal tonsils and kidney were collected individually and frozen at -70°C for virus detection by RT-PCR. In addition, pieces of trachea and kidney were fixed in 10% buffered formalin for histopathology. Blood samples were collected from 8 randomly selected chicks at 0, 15, 21and 28 dpi from the SPF chicks and at 0, 3, 6, 9, 15, 21 and 28 dpi from the broiler chicks to monitor antibody responses.

3.4. 3. Gross and microscopic lesions

All euthanised and found dead chicks were necropsied and examined for gross lesions. The trachea and kidney were scored individually according to the criteria described in Chapter 2.5.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. Trachea and kidney lesions were examined and scored as follows: 0 = no change, 1= mild, 2 = moderate, 3 = severe (Chen et al., 1996).

3.4.4. Swab and tissue sample processing for RT-PCR

Each set of swabs (either OP or CL) were dipped and pooled as a single sample in 1.5 ml of TOC medium (Appendix 1). Individual tissue samples of tracheal, lung, kidney and caecal tonsils were ground up using a pestle and mortar (Chapter 2.9.iii). RNA for the two experiments was extracted from swab and tissue samples using the QIAamp viral RNA mini kit following manufacturer's instructions (Chapter 2.10.ii). RT-PCR was carried out as described in Chapter 2.10.iii.

3.4.5. Serological assay

Sera were tested using commercial ELISA kits (IDEXX) according to the manufacturer's instructions (Chapter 2.12.i.b). HI testing was performed using 4 HAU as described in Chapter 2.12.ii. The virus strains used as antigens for the HI test were IBV M41, 793B and IS/885. IBV HI titres were expressed as $\log_2$ values of the highest reciprocal of the dilution that showed HI.

3.4.6. Statistical analysis

Data for gross and histopathological lesions and ELISA were analysed using the Mann-Whitney *U* test. The mean HI antibody titres of infected SPF and broiler chicks sera tested with different IBV antigens were compared and analysed using one way ANOVA to test for a significant overall effect, followed by Tukey's test to identify which means were significantly different from each other. All analysis were conducted using GraphPad Prism, 6.0.1

3.5. Results

3.5. 1. Clinical signs

No clinical signs or mortalities were observed in the control groups of either experiment.

In the SPF chicks, clinical signs were first observed at 1 dpi, which included mild tracheal râles, sneezing, coughing, head shaking and eye scratching. Gasping, wheezing and open mouth breathing were seen in some cases (4-5 birds) between 4 to 13 dpi (Figure 3.1 A) and wet dropping (white or milky faeces) were also observed. All clinical signs disappeared completely by 18 dpi. Of 45 chicks, one bird died at 8 dpi (2.2%).

For the commercial broiler chicks, the clinical signs were similar to those observed in SPF chicks but with a lower severity and lasting for a longer period. Clinical signs were first observed at 3 dpi and the last at 22 dpi. Of the 45 birds, three died; one at 12 dpi and two at 19 dpi (6.6%). Apart from respiratory signs, one bird showed swelling of the head, foamy eyes and nasal discharge at 14 dpi. The swelling increased at 15 dpi, with the periocular tissues particularly affected (Figure 3.1, B). For welfare reasons, this bird was euthanized for necropsy and sampling was carried out.

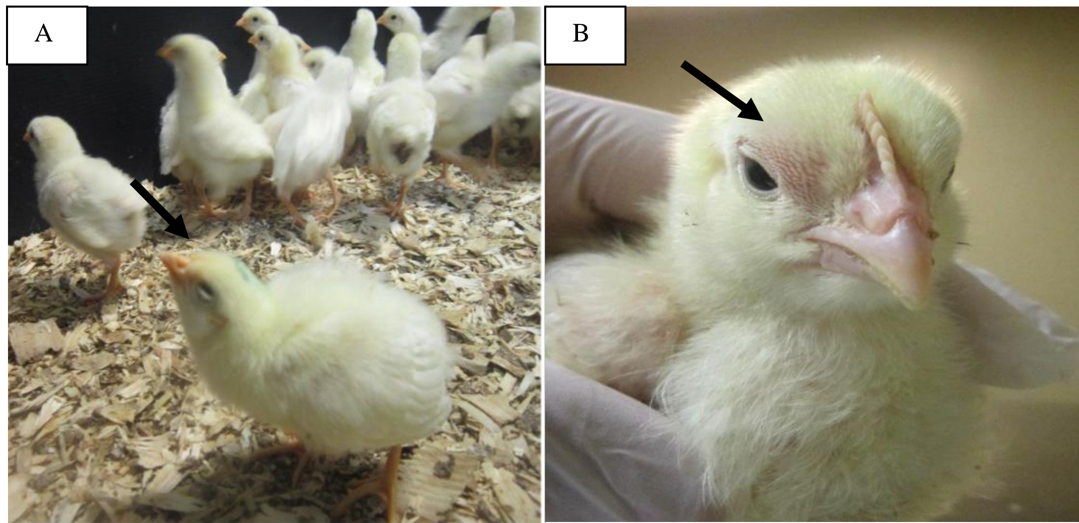

Figure 3.1. A, Respiratory distress, gasping and open-mouth breathing of an infected SPF chick (Arrow). B, Periocular swelling of an infected broiler chick at 15 dpi (Arrow).

3.5. 2. Gross necropsy findings

No detectable gross lesions were witnessed in the two control groups. A summary of all gross lesions seen in the infected SPF and broiler chicks is presented in Table 3.1. In the SPF chicks (Expt 1), tracheal lesions mostly comprised congestion and presence of excess mucus (observed from 3 dpi onward). Pale kidneys with swelling and large amounts of urate deposits were observed at 6 dpi (Figure 3.2, B). These lesions were not seen after 12 dpi (Table 3.1). At 28 dpi, (during the post mortem examination) water-like fluid accumulation was found in the oviduct of two female SPF chicks (Figure 3.2, C). Samples of the fluid and tissues of the cystic oviduct were collected for virus detection. In addition, tissue sample from the cystic oviduct was collected for histopathology. The fluid was positive for IBV by RT-PCR and VI, while the tissue was negative. Part-sequencing of the hypervariable region of the S1 gene of this isolate revealed 99% homology with the challenge virus of IS/885

that was used for infection. Necropsy of the dead bird at 8 dpi showed large swollen, pale kidneys with tubules and ureters distended with urates.

In the broiler chicks (Expt 2), the gross lesions of the trachea and kidney were similar to those observed in Expt 1 but with lower severity. However, the lesions persisted for a longer time than in the SPF chicks (Table 3.1). Severe tracheal lesions such as plugs of yellow caseous were observed at 15 dpi (Figure 3.2, A). Paleness and swelling of kidneys were observed from 6 to 21 dpi. However, there was no significant difference regarding the extent and severity of the kidney lesions between the two infected groups. The only exception was at 6 dpi, where the severity of kidney lesions of the SPF chicks was significantly higher ($P = 0.03$) than those in the broilers.

The chick with swelling of the head was euthanized at 15 dpi. Subcutaneous swabs were taken for virus detection (IBV and aMPV) by RT-PCR, VI and for bacterial culture (*E. coli*). Clear positive signal was obtained for IB virus. DNA sequence of the par-S1 gene fragment obtained was 100 % identical with the IS/885 strain (the inoculum). Isolation in SPF ECEs yielded a positive result at the third passages, with typical lesions including dwarfing and curling of the embryos. Neither aMPV nor *E. coli* was detected in the swabs. Necropsy of birds that died showed emaciation, dehydration, cheesy exudate plugs in the upper trachea and pale, swollen kidneys. In addition they had fibrinous pericarditis, fibrinous perihepatitis, airsacculitis and peritonitis (Figure 3.2, D).

Table 3.1. Gross lesions of the trachea and kidney of SPF or broiler chicks infected with IBV IS/885 strain.

	SPF		Broiler	
dpi	**Trachea**	**Kidney**	**Trachea**	**Kidney**
3	0.4±0.2 (2)	0.0±0.0	0.4±0.2 (2)	0.0±0.0
6	0.6±0.2 (3)	1.6±0.4[a] (5)	0.6±0.2 (3)	0.4±0.2[b] (3)
9	1.2±0.2 (5)	0.8±0.3 (3)	0.6±0.2 (3)	0.6±0.2 (3)
12	1.4±0.4 (4)	0.4±0.2 (2)	1.0 ±0.3 (4)	0.8±0.2 (4)
15	0.0±0.0	0.0±0.0	1.2±0.5 (3)	0.6±0.2 (3)
21	0.0±0.0	0.0±0.0	0.4±0.2 (2)	0.4±0.2 (2)
28	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Data are expressed as the mean gross lesion score ± SEM. For each time point and tissue, different superscript letters for SPF and broiler indicate they are significantly different ($p < 0.05$). Absence of letter indicates that there were no significant differences between the lesions of infected groups ($p > 0.05$). Numbers in parenthesis are number of chicks with gross lesion, out of five examined.

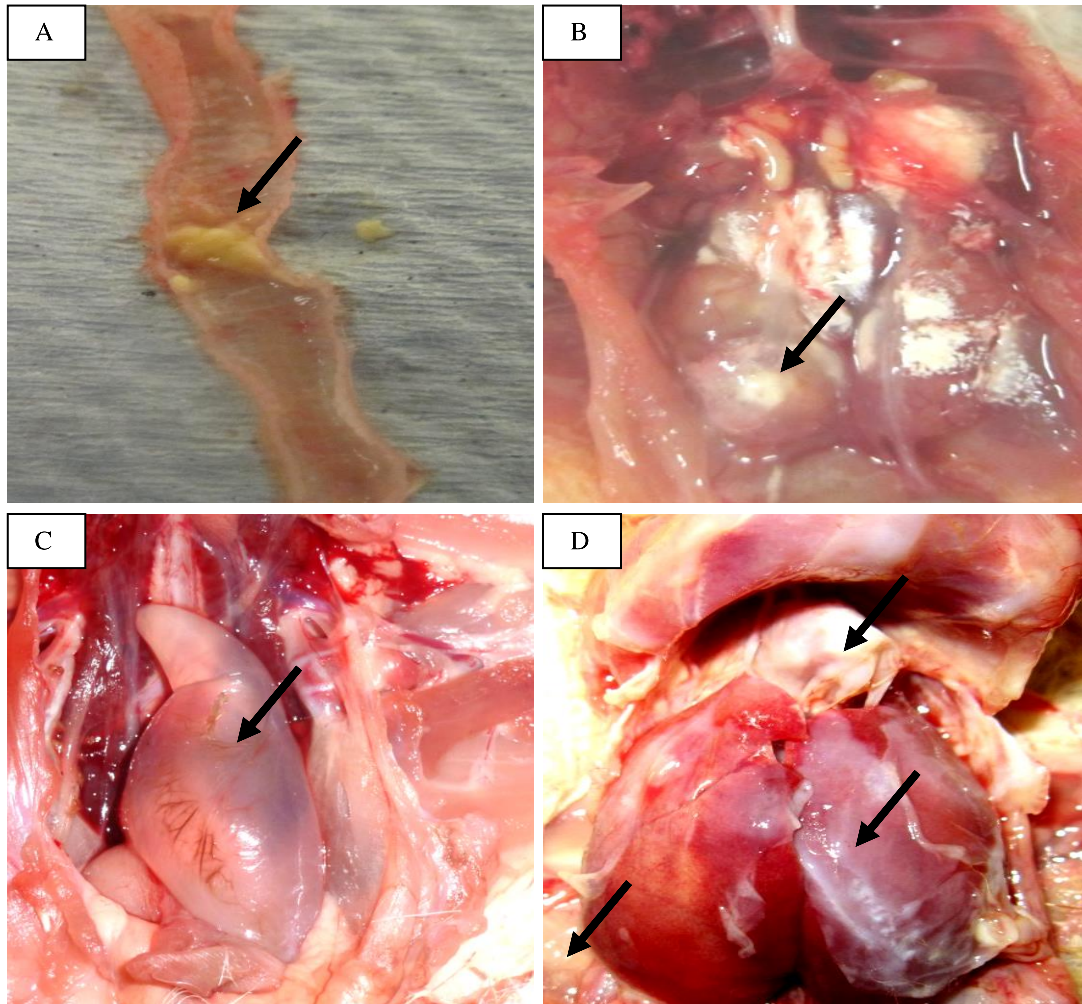

Figure 3. 2. A, Mucoid exudate and caseous plug in the trachea of an infected broiler chick at 15 dpi. B, large swelling of kidneys, pale tubules and ureters distended with urates of an infected SPF chick at 6 dpi. C, Cystic dilation of the oviduct of an SPF female chick at 28 dpi. D, Fibrinous pericarditis, fibrinous perihepatitis and peritonitis in an infected broiler chick which died at 12 dpi.

3.5. 3. Histopathology

No histological abnormalities were observed in the trachea (Figure 3.3, A), kidneys (Figure 3.3, D) or oviduct of the control groups in either experiment.

3.5. 3.1. Trachea of infected chicks

Details of histological lesions in the tracheas in both experiments are summarised in Table 3.2. In both lines of chicks (SPF and broiler) they were essentially the same; however the severity differed throughout study period.

In the SPF chicks, severe changes in the trachea were observed at 3 dpi. The most consistent lesions in the SPF chick were loss of cilia (Figure 3.3, B) and heterophil infiltration, decreased mucous cells, and occasional heterophilic exudate in the tracheal lumen. Mild to moderate lesions were witnessed until 15dpi. Ciliated epithelium redeveloped at 21dpi. Areas of severe lymphoid infiltration in the lamina propria or submucosa persisted until the end of the experiment (Table 3.2).

In the broilers chicks, at early stage of infection, each lesion type was less severe than those observed in the SPF chicks but persisted for longer (Figure 3.3, C). At 21 dpi, the histopathological changes were significantly greater ($p < 0.05$) than those observed in the SPF chicks (Table 3.2).

3.5. 3.2. Kidney of the infected chicks

A summary of the histopathological changes in the kidneys during both experiments is given in Table 3.3.

In the SPF chicks, kidney lesions developed by 3 dpi, which included ducto-tubular dilation, interstitial heterophilic infiltration and epithelial hyperplasia. The main histological lesions consisted of interstitial lymphoid infiltration with mild lymphoid nodules observed throughout the study. Most of the kidney lesions had cleared by 21

dpi, apart from mild lymphoid infiltration and lymphoid nodules that were present until the end of the experiment (Table 3.3).

In the broiler chicks, kidney lesions were first observed at 6 dpi, which included tubular degeneration and hyperplasia of the epithelium. Lymphoid infiltration was the main lesion observed throughout the observation period. No significant differences were observed among the histological changes regarding the extent and severity of the lesions between the SPF and broiler chicks.

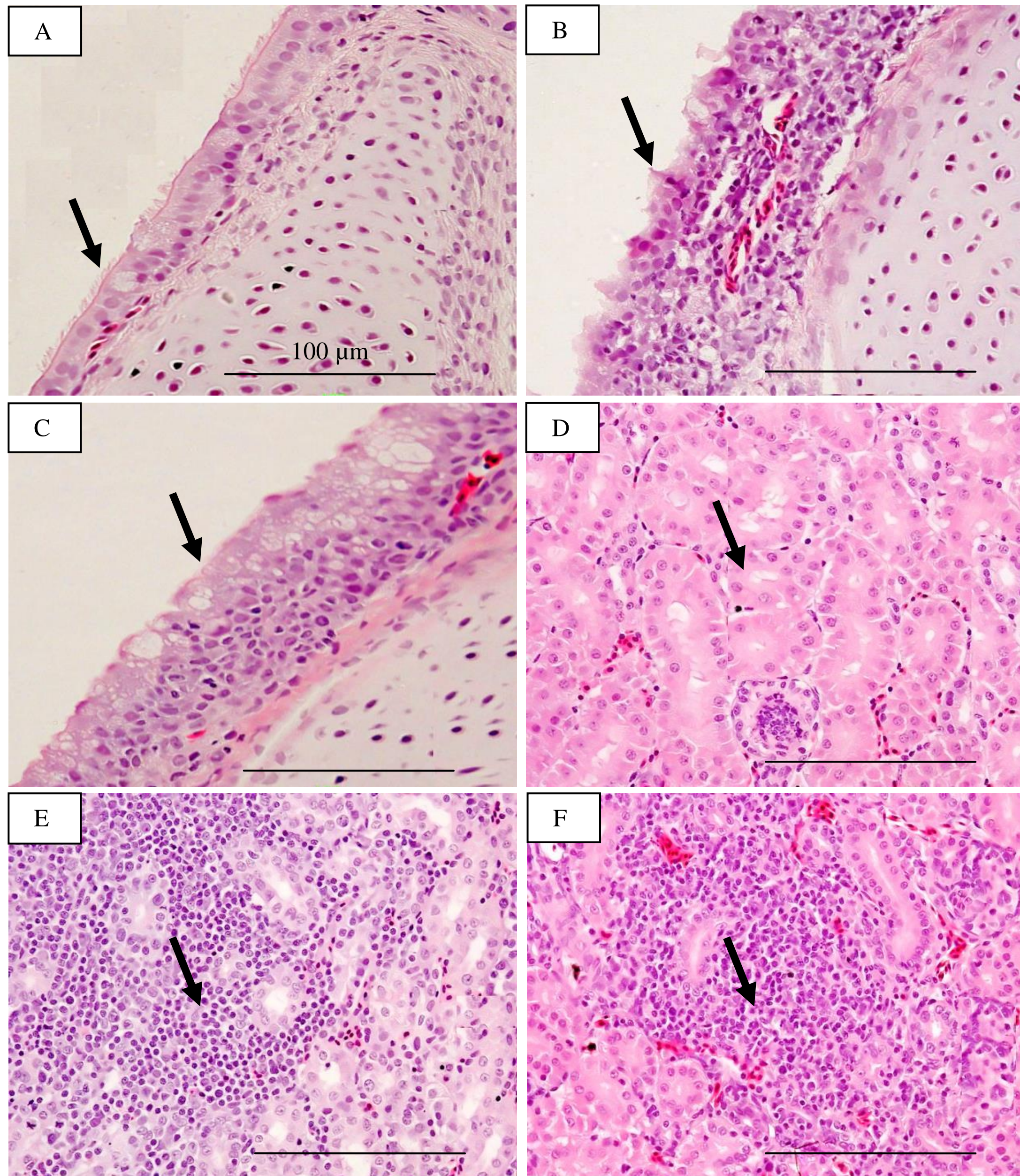

Figure 3.3. Hematoxylin and eosin stains of the trachea and kidney (magnification at 400x). Panels A (trachea) and D (kidney) correspond to control chick tissue taken at 3 and 9 days of age respectively. B, Extensive epithelial deciliation, with severe lymphocyte and heterophil infiltration of trachea of SPF chicks at 3 dpi. C, Mild epithelial deciliation, with moderate lymphocyte and heterophil and mild epithelial hyperplasia of broiler chicks at 3 dpi. E, Moderate to severe lymphocyte and mild heterophil interstitial infiltration in kidney of SPF chicks at 9 dpi. F, Mild to moderate lymphocyte and heterophil interstitial infiltration in kidney of broiler chick at 9dpi.

Table 3.2. Histopathology of the trachea in SPF or broiler chicks infected with IBV IS/885 strain.

Tracheal lesions	SPF (dpi)							Broiler (dpi)						
	3	**6**	**9**	**12**	**15**	**21**	**28**	**3**	**6**	**9**	**12**	**15**	**21**	**28**
Epithelial deciliation	3.0±0.0^{a}	ND	3.0±0.0	2.8±0.2	0.5±0.2	0.0±0.0^{a}	0.0±0.0	1.6±0.4^{b}	2.8±0.2	2.4±0.2	2.4±0.4	2.2±0.4	2.0±0.3^{b}	0.0±0.0
Epithelial degeneration	0.5±0.2	ND	1.2±0.2	1.0±0.3	1.0±0.4	0.5±0.2	0.5±0.5	0.8±0.2	1.2±0.2	1.4±0.2	1.8±0.3	1.0±0.0	1.4±0.2	0.2±0.2
Decrease mucous cells	1.2±0.2^{a}	ND	2.2±0.2	2.6±0.2	2.0±0.4	1.2±0.5	1.5±0.5	0.2±0.2^{b}	1.8±0.5	1.8±0.2	2.0±0.3	2.8±0.2	2.2±0.3	0.5±0.2
Heterophil infiltration	2.0±0.0^{a}	ND	0.5±0.2	0.8±0.3	0.0±0.0	0.0±0.0^{a}	0.0±0.0	0.8±0.3^{b}	0.8±0.3	1.6±0.5	1.0±0.3	0.6±0.2	1.2±0.3^{b}	0.2±0.2
Epithelial hyperplasia	1.7±0.2	ND	2.7±0.2^{a}	2.6±0.2^{a}	1.2±0.2	1.0±0.0^{a}	1.5±0.5	1.2±0.2	1.4±0.2	1.6±0.2^{b}	1.0±0.0^{b}	1.0±0.4	2.0±0.0^{b}	1.5±0.2
Lymphoid infiltration	2.0±0.0^{a}	ND	1.5±0.2	1.4±0.2	2.0±0.0	1.7±0.2	2.0±0.0	1.0±0.0^{b}	1.2±0.2	1.8±0.2	1.6±0.2	1.6±0.4	2.2±0.2	1.5±0.2

ND: not done; 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). For each time point and lesions, different superscripts for SPF and broiler chicks indicate significant differences between them ($p < 0.05$). Absence of a letter indicates that there were no significant differences ($p > 0.05$).

Table 3.3. Histopathology of the kidney in SPF or broiler chicks infected with IBV IS/885 strain.

Kidney lesions	SPF (dpi)							Broiler (dpi)						
	3	**6**	**9**	**12**	**15**	**21**	**28**	**3**	**6**	**9**	**12**	**15**	**21**	**28**
Epithelial degeneration	0.0±0.0	0.0±0.0	0.6±0.2	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.4±0.2	0.0±0.0	0.0±0.0	0.2±0.2	0.2±0.2	0.0±0.0
Ducto-tubular dilation	0.4±0.2	0.4±0.2	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.2	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0
Heterophil infiltration	0.2±0.2	1.0±0.0[a]	0.2±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0[b]	0.2±0.2	0.0±0.0	0.2±0.2	0.0±0.0	0.4±0.2
Lymphoid infiltration	0.0±0.0	0.0±0.0	1.0±0.0	1.6±0.2	1.0±0.0	0.6±0.2	0.4±0.2	0.0±0.0	0.0±0.0	1.0±0.0	1.2±0.2	0.6±0.2	0.6±0.2	0.0±0.0
Epithelial regeneration	0.0±0.0	0.0±0.0	0.2±0.2	0.2±0.2	0.8±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.8±0.3	0.2±0.0	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0
Epithelial hyperplasia	0.2±.02	0.0±0.0	0.6±0.2	0.2±0.2	1.0±0.0[a]	0.0±0.0	0.0±0.0	0.0±0.0	0.2±0.2	0.0±0.0	0.0±0.0	0.2±0.2[b]	0.2±0.2	0.0±0.0
Lymphoid nodules	0.0±0.0	0.0±0.0	0.6±0.2	0.8±0.2	1.0±0.0[a]	0.4±0.2	0.8±0.2	0.0±0.0	0.0±0.0	0.0±0.0	0.6±0.4	0.0±0.0[b]	0.4±0.4	1.0±0.0

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). For each time point and lesions, different superscripts for SPF and broiler chicks indicate significant differences between them ($p < 0.05$). Absence of a letter indicates that there were no significant differences ($p > 0.05$).

3.5. 3.3. Oviduct of infected SPF chicks

The oviduct lumen is multifocally partially lined by compact epithelial cells which have entirely lost their cilia (Figure 3.4). Many epithelial cells are shed into the lumen leaving sections devoid of mucosa. The underlying submucosa is multifocally infiltrated by both moderate numbers of degenerate and viable lymphocytes and plasma cells with lesser numbers of macrophages.

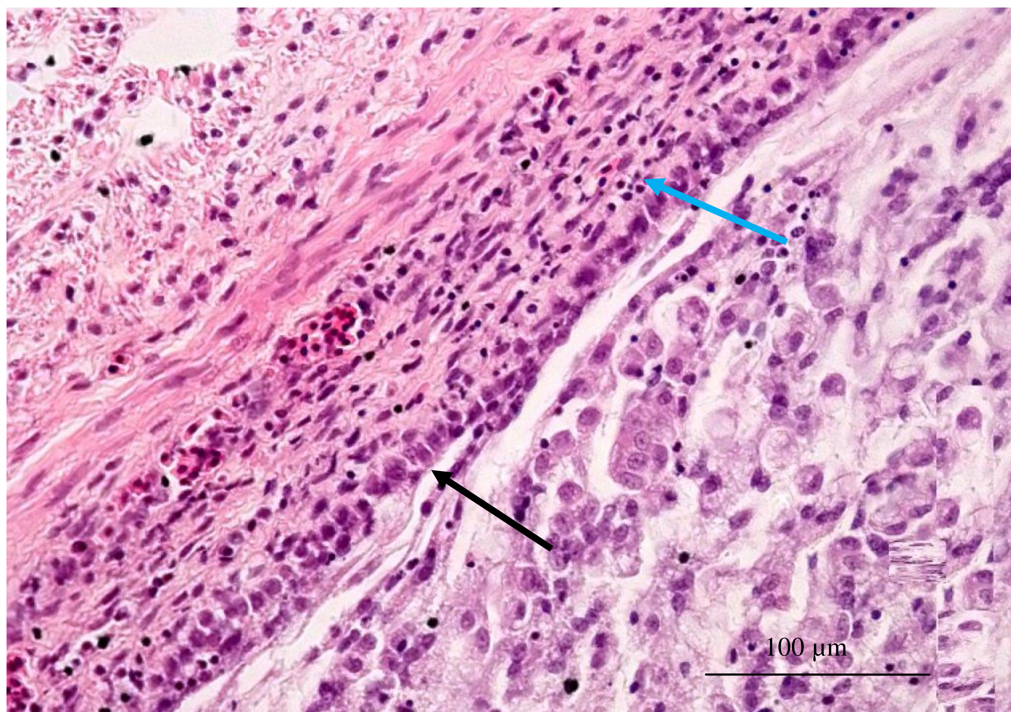

Figure 3.4. Hematoxylin and eosin stains of the cystic oviduct of infected female SPF chicks at 28dpi (magnification at 400x). Multifocal moderate chronic lymphocytic salpingitis with cystic dilation. Diffuse loss in the cilia (Black arrow) and infiltration of lymphocytes and plasma cells (Blue arrow).

3.5.4. IBV RT-PCR

No virus was detected in the control groups of either experiment throughout the study. The viral genome was detected in pooled OP and CL swabs (Table 3.4) from both infected groups for the duration of the study (28 dpi).

In the SPF chicks, the virus was detected in the trachea, lung, caecal tonsil and kidney in most of the tested samples for up to 15 dpi. Beyond that, the virus was infrequently detected in tissues (Table 3.5).

In the broiler chicks, detection of the virus was lower than observed in the SPF chicks throughout the observation period. It was found most frequently in the caecal tonsil, followed by trachea (Table 3.5).

Table 3.4. Virus detection by RT-PCR in swabs of SPF or broiler chicks infected with IBV IS/885 strain.

dpi	**SPF**		**Broiler**	
	OP	**CL**	**OP**	**CL**
3	+	+	+	+
6	+	+	+	+
9	+	+	+	+
12	+	+	+	+
15	+	+	+	+
21	+	+	-	+
28	+	+	+	+

Table 3.5. Virus detection by RT-PCR in tissue of SPF or broiler chicks infected with IBV IS/885 strain.

dpi	SPF				Broiler			
	Tr	**L**	**CT**	**kid**	**Tr**	**L**	**CT**	**kid**
3	5*	5	4	4	3	1	5	5
6	5	5	5	5	5	2	5	2
9	5	5	5	5	2	0	4	0
12	5	5	4	5	4	1	2	1
15	5	5	5	5	4	2	4	1
21	2	3	4	3	0	0	3	1
28	1	3	3	0	1	0	5	2
Total≠	28	31	30	27	19	6	28	12

** Values presented are number of IBV-positive cases out of five birds examined. Tr= Trachea, L= lung, CT= caecal tonsil, Kid= kidney. ≠ Total number of tissues that was positive for IBV.*

3.5.5. Serology

3.5.5. 1. ELISA

Analysis of the antibody titres of both experiments using ELISA is summarised in Table 3.6.

In the SPF chicks, sera collected prior to experimental infection were free of IBV antibodies. Chicks which received virus-free AF had ELISA titres lower than the detectable level throughout the experimental period. In the IS/885-infected chicks, an increase of antibody titres occurred at 21 dpi which decreased by 28 dpi (Table 3.6).

In broiler chicks, high levels of IBV MDA were detected in all birds at 1-day-old. By 3 dpi, the MDA had declined in the control and infected groups and showed no significant differences (Table 3.6). At 9 and 15 dpi, neither the infected nor control group had antibody titres above the cut-off point. However, at 21 and 28 dpi, highly significant antibody titres were observed in the infected group compared to the control group (Table 3.6).

Table 3.6. Mean ELISA antibody titres in the SPF and broiler chicks infected with IBV IS/885 strain.

	SPF		Broiler	
dpi	**Infected**	**Control**	**Infected**	**Control**
0	21±9.0	21±9.0	2867±229	2867±229
3	ND	ND	1813±343	1809±544
6	ND	ND	531±153	501±64
9	ND	ND	311±85	282±75
15	160±64[a]	4.0±2.0[b]	378±175	75±19
21	551±146[a]	12±5.0[b]	1116±129[a]	19±8.0[b]
28	481±194[a]	7.0±4.0[b]	623±35[a]	10±8.0[b]

ND: test not done. Data are expressed as the mean values ± SEM (n = 8). Data in the same row with different superscript letters are significantly different in antibody titres (p < 0.05). Absence of a letter indicates that there were no significant differences (p > 0.05) between any of the time points. The cut-off point = 396.

3.5.5. 2. HI

Analysis of the HI antibody titres of both experiments using different IBV antigens are summarised in Tables 3.7 and 3.8.

In the SPF chicks, all sera collected prior to experimental infection and from the control group were free of IBV HI antibodies (Table 3.7). At 15 dpi, the HI titres against the heterologous antigens of M41 and 793B were low ($<3 \log_2$) compared to the homologous antigen ($3.8 \log_2$). At 21 dpi, chicks showed high antibody titres ($\geq 3 \log_2$) compared to control birds. The birds showed high significant level ($p < 0.05$) of IBV HI antibody titres when IS/885 was used as antigen throughout the sampling time (Table 3.7).

In the broiler chicks, sera collected at one day old showed a high, significant antibody titre when either M41 or 793B were used as the antigen (Table 3.8). Titres had started to decline significantly by 3 dpi in both the infected and control groups. At 6 and 9 dpi, there were no detectable titres in either ($<3 \log_2$). When all IBV antigens were compared, higher levels of IBV antibody titres were seen when IS/885 used as the antigen from 15 dpi onwards.

Table 3.7. Mean HI antibody titres in the SPF chicks infected with IS/885 strain using homologous and heterologous antigens.

dpi	Infected			Control		
	M41	**793B**	**IS/885**	**M41**	**793B**	**IS/885**
0	0.6±0.2	1.1±0.2	0.7±0.2	0.6±0.2	1.1±0.2	0.6±0.2
3	ND	ND	ND	ND	ND	ND
6	ND	ND	ND	ND	ND	ND
9	ND	ND	ND	ND	ND	ND
15	1.5±0.3[a]	0.6±0.1[a]	3.8±0.2[b*]	1.1±0.1	0.6±0.1	0.6±0.1
21	4.0±0.2[a*]	3.6±0.3[a*]	5.1±0.3[b*]	0.8±0.3	0.7±0.2	0.3±0.1
28	2.5±0.4[a*]	2.6±0.1[a*]	3.9±0.2[b*]	0.6±0.1	0.2±0.1	0.3±0.2

ND: test not done. Data are expressed as the mean values ± SEM (n = 8). Data in the same row across with different superscript letters are significantly different ($p < 0.05$). Absence of a letter indicates that there were no significant differences ($p > 0.05$) between the IBV antigens at any of the time points. [*]Values differed significantly from each representative control group.

Table 3.8. Mean HI antibody titres in the broiler chicks infected with IS/885 strain using IBV homologous and heterologous antigens.

	Infected group			**Control**		
dpi	**M41**	**793B**	**IS/885**	**M41**	**793B**	**IS/885**
0	6.2±0.6 [a]	5.8±0.5[a]	3.5±0.2[b]	6.2±0.6[a]	5.8±0.5[a]	3.5±0.2 [b]
3	5.1±0.7[a]	5.0±0.2[a]	2.6±0.1[b]	5.1±1.1[a]	5.5±0.8[a]	3.1±0.1[b]
6	2.4±0.6	1.3±0.3	2.1±0.2	3.0±0.3 [a]	3.3±0.5[b]	1.7±0.3[a]
9	1.6±0.8	1.4±0.2	1.8±0.2	1.6±0.8	2.3±0.6	1.1±0.2
15	3.5±0.1[a*]	3.3±0.1[a*]	4.0±0.2[a*]	1.3±0.4	0.7±0.1	1.0 ±0.0
21	5.0±0.0 [a*]	4.2±0.4[a*]	5.8±0.2[b*]	1.1±0.3	0.6±0.1	0.3±0.2
28	3.7±0.1[a*]	3.5±0.1[a*]	4.5±0.1[b*]	0.7±0.2	0.4±0.1	0.1±0.1

Data are expressed as the mean values ± SEM (n = 8). Data in the same row across with different superscript letters are significantly different (p < 0.05). Absence of a letter indicates that there were no significant differences (p > 0.05) between the IBV antigens at any of the time points. [*]Values differed significantly from each representative control group.

3.6. Discussion

The results presented in this study demonstrate that IS/885/00 is a virulent IBV, as extensive disease was produced following infection in both types of chicks. In addition to the respiratory signs, gross lesions comprising tracheal caseous exudate and plugs, and swollen kidneys with (or without) urate deposits were observed. Such clinical signs have been observed following infection with virulent IBV M41(Butcher et al., 1990), Moroccan G strain (Ambali and Jones, 1990),793B (Boroomand et al., 2012), It-02 (Dolz et al., 2012) and QX (Ganapathy et al., 2012). These clinical signs and lesions were more severe in SPF than in the broiler chicks, indicating differences in the susceptibility to IBV infection in these different types of birds. Such differences could be contributed to the genetic line of the birds or the IBV MDAs in the broiler chicks (Ignjatovic et al., 2003). Previous studies using virulent IBV M41 demonstrated a considerable difference between two white leghorn chick lines in term of the severity and duration of respiratory signs (Otsuki et al., 1990). Another study compared mortalities in several inbred lines of chickens following inoculation with a pool of strains of IBV and/or *E.coli* and found marked differences among them (Bumstead et al., 1989).

It has been reported that chicks with high antibody titres had excellent protection against infection by IBV M41 at one day old but not at seven days old (Mondal and Naqi, 2001) and that MDA does not prevent the viral infection but does reduce the pathogenic effects of the IBV infection in young chicks (Klieve and Cumming, 1988). Our findings suggest that MDA is an important factor in determining the severity of the disease caused by IBV IS/885 strain at a young age.

Following IS/885 infection of the broiler chicks, clear periorbital swelling was noted in one bird at 15 dpi. The swab collected from this site was positive for IBV by RT-PCR and VI, and sequencing demonstrated 100% nucleotide level similarity to IS/885. Neither aMPV nor bacteria were detected or cultured from swabbing, suggesting that mild head swelling was due to the IBV infection. This finding is similar to the first report of SHS where untyped coronavirus and *E.coli* were isolated from a broiler chickens in Southern Africa (Morley and Thomson, 1984). IBV M41 and *E.coli* were isolated from broiler flock in the USA that experienced SHS (Droual and Woolcock, 1994). Apart from IBV, aMPV (Picault et al., 1987; Aung et al., 2008), FAdV (Droual and Woolcock, 1994; Georgiades et al., 2001) and the presence of a secondary infection (such as *E.coli*) have been implicated as the cause of SHS (Nakamura et al., 1997; Nakamura et al., 1998).

In the IS/885-infected SPF chicks, necropsy examination at 28 dpi revealed dilation of the oviduct with fluid content (cystic oviduct) in two female chicks. Cystic oviduct formation following infection of virulent IBVs at a young age has been reported for IBV variants M41 (Crinion et al., 1971; Crinion and Hofstad, 1972a; Jones and Jordan, 1972) and recently, QX (Benyeda et al., 2009; de Wit et al., 2011b; Ganapathy et al., 2012). We report, for the first time, similar pathological development in SPF chicks that received the IS/885 virus at 1-day-old. In addition, the virus with 99% part-S1 sequence similarity to IS/885 was isolated from the cystic fluid. The epithelial cells of the oviduct are the primary cells attacked by the IBV (Crinion and Hofstad, 1972a). From this evidence, the IS/885 has caused a diffuse loss in the cilia, epithelial necrosis and infiltration of lymphocytes and plasma cells into the oviduct which indicates that the virus has replicated in the epithelial cells lining the oviduct.

The pathogenicity of IBV strain for the oviduct varies as some do not replicate in the oviduct neither causing lesions nor showing significant amounts of viral antigen in the epithelial cells (Crinion and Hofstad, 1972a). For instance, IBV Conn and Iowa failed to produce any gross or histopathological change in the oviduct which involved incidence of microscopic cystic oviduct and cellular infiltration and oedema in the oviduct wall. In contrast, IBV M41 produced the greatest number of changes, followed by Australian T which both showed significant amounts of IBV antigen by immunofluorescence in epithelial cells lining the oviduct (Crinion and Hofstad, 1972a). Benyeda et al. (2009) also did not detect any abnormalities in the oviducts of 793B infected 1-day-old SPF chicks, whereas a variable percentage of oviduct dilatations were detected following inoculation with five different QX-like strains. It appears that along with M41 and QX, IS/885 should be considered as a potential cause of cystic oviducts in chickens.

The microscopic findings in the SPF and broiler chicks following IS/885 infection were similar to previous reports (Albassam et al., 1986; Nakamura et al., 1991; Chen et al., 1996; Benyeda et al., 2010). In this work, the SPF chicks had greater degeneration in the trachea, indicating a more extensive virus replication than broiler chicks, which may have been due to extravasation of circulating maternal antibody in the latter. In both types of birds, severe and early onset of histopathological lesions was seen in the trachea, compared to mild lesions in the kidney. Nephropathogenic IBVs have shown tropism for respiratory and kidney tissues although kidney lesions are more apparent (Albassam et al., 1986; Butcher et al., 1989). Based on our findings, the IS/885 strain appears to have an affinity for both respiratory and renal tissues.

In the current study, persistence of the viral genome was observed in the selected tissues and swabs until the end of all experiments (28 dpi). Previous studies have reported the detection of virulent IBV It-02 in the trachea and kidney of infected SPF chicks by RT-PCR for up to 21 dpi (Dolz et al., 2012). For other pathogenicity studies conducted in broiler chicks free of MDA, the viral genome of virulent IBV 793B was detected in the trachea for up to 13 dpi and in the kidney for up to 15 dpi (Boroomand et al., 2012). Discrepancies in these findings could be due to individual IBV strain differences. The viral genome could be detected in the kidney as early as 3 dpi. Such observations are supported by several other studies in which virulent IBV 793B (Boroomand et al., 2012) and It-02 (Dolz et al., 2012) were detected by RT-PCR from the kidney of infected SPF chicks from 1 dpi. Dhinakar Raj and Jones (1996a) reported the isolation of 793B from the kidney of infected SPF chicks at 1 dpi and from broiler chicks at 3 dpi.

Our findings showed that the total amount of positive tissue in the SPF chicks was greater than in the broilers. This may relate to the extensive virus replication in fully susceptible chicks. This is similar to the finding of Dhinakar Raj and Jones (1996a), where virulent 793B was recovered from respiratory tissues (trachea and lung) for 7 to 10 dpi in SPF chicks but only 3 dpi in broilers.

Virus shedding in both OP and CL was investigated by RT-PCR and the viral genome was consistently detected until the end of the experiment. A similar observation was reported, in which IBV It-02 was detected by RT-PCR in nasal and CL swabs for up to 27 dpi (Dolz et al., 2012). Previously, El Houadfi *et al.* (1986) reported the isolation of the Moroccan G strain from CL swabs for up to 28 dpi. These observations indicate the virus persisted for longer period in the OP and CL swabs.

The immune response after infection with IBV was measured by ELISA and HI. High level of maternal antibodies at 0, 3 and 6 days old for IBV could be detected by HI when IBV M41 and 793B were used as antigens compared to IS/885. These findings may due to the parent flock of the broiler chicks having been vaccinated with Mass or/and 793B types.

ELISA detected low to moderate levels of the antibody to IBV in the infected SPF chicks, compared to infected broilers. This discrepancy could be related to different types of birds. However, significant levels of HI antibodies against the IS/885 antigen were witnessed when compared to the level of antibody response against M41 and 793B antigens. This finding may reflect the use of the homologous virus as an antigen in the HI test, as it is well known that the HI test is strain specific (Monreal et al., 1985; de Wit et al., 1997).

In conclusion, this study showed that IBV IS/885 is pathogenic for both SPF and broiler chicks under experimental conditions. The virus affected epithelium of the trachea and produced severe kidney lesions; however, the disease was more severe in SPF than in the broiler chicks. Our study suggests the IS/885 has tropism for both respiratory and renal tissues. In addition, it could be implicated in the pathology of head-swelling and cystic oviduct development in young chicks.

Chapter 4: Protection conferred by live infectious bronchitis vaccine viruses against variant Middle East IS/885/00-like and IS/1494/06-like isolates in commercial broiler chicks

4.1. Abstract

The ability of the IB H120 (a Massachusetts strain) and CR88 (a 793B strain) live attenuated vaccine viruses to protect against two Middle East isolates IS/885 and IS/1494/06-like (IS/1494) in broiler chicks was investigated. Day-old chicks were separated into three groups, I) vaccinated with H120 at day-old followed by CR88 at 14 days-old, II) vaccinated with H120 and CR88 simultaneously at day-old and again with CR88 at 14 days-old, III) control unvaccinated. At 30 days-old, each of the groups was challenged with virulent IS/885 or IS/1494. Protection was evaluated based on the clinical signs, tracheal and kidney gross lesions, tracheal ciliary scores and virus detection by RT-PCR. Results showed that administering combined live H120 and CR88 vaccines simultaneously at day old followed by CR88 vaccine at 14 days-old gave more than 80% cilliary protection against both of the Middle East isolates. In addition, this programme conferred 100% protection against clinical signs and tracheal/kidney lesions. The other vaccination programme, H120 at day-old followed by CR88 at 14 days-old, provided somewhat lower protection, in particular against IS/885.

4.2. Introduction

IB is a ubiquitous virus with a high mutation rate, and a large number of serotypes or genotypes of IBV strains have been reported worldwide (de Wit et al., 2011a). Variant strains emerge due to changes in the IBV genome through point mutations, deletions, insertions or RNA recombination and these variants are often responsible for IB outbreaks in vaccinated chicken flocks (Cavanagh et al., 1988; Jia et al., 1995; Liu et al., 2007). Many countries have shown that multiple variant IBV strains are circulating in their poultry flocks (de Wit et al., 2011a).

IS/885/00 and IS/1494/06 or those with high similarities to these strains of IBVs have been reported throughout the Middle East and North Africa (Meir et al., 2004; Mahmood et al., 2011; Abdel-Moneim et al., 2012; Kahya et al., 2013; Awad et al., 2014). In most cases, severe respiratory distress and renal lesions with high mortality were observed in vaccinated flocks affected by these strains. It appears that the conventional H120 vaccines alone do not provide sufficient protection against these strains (Meir et al., 2004; Kahya et al., 2013). However, development of vaccines against each of these new variants is not generally an option due to the high cost and time required for product registration (Jackwood et al., 2003; Bijlenga et al., 2004). Furthermore, it could be a never-ending race as new variant IBVs are constantly emerging in major poultry producing countries. Instead, it has been recommended that protection conferred by available live IBV vaccines against new variants should be constantly evaluated (Alvarado et al., 2003). Vaccination with one serotype does not ensure complete protection against heterologous strains (Cook et al., 1999a) but the use of different combinations of live IBV vaccines has been shown to be able to induce high and broad protection against challenges with several heterologous

virulent IBV variants (Gelb et al., 1991; Cook et al., 1999a; Cook et al., 2001a; Alvarado et al., 2003; Martin et al., 2007; Terregino et al., 2008).

The objective of this study is to evaluate the protection conferred by available live IBV vaccines when used in strategic manner against the two prominent Middle East variant IBVs that are related to IS/885/00 and IS/1494/06. In addition to the conventional vaccination programme (Mass at day-old followed by variant at 14 days-old); we also evaluated another vaccination programme where the Mass and variant live vaccines were given simultaneously at day-old followed by a variant vaccine at 14 days-old. Following challenge, the protection was assessed based on ciliostasis assay as recommended by European Pharmacopoeia. In addition, clinical signs, gross lesions and presence of the viral genome in the trachea and kidney were evaluated.

4.3. Materials and methods

4.3.1. Chicks

Day-old commercial broiler chicks with IBV MDA were used in this study (Chapter 2.3.ii). Chicks were kept in an isolation unit throughout the experiment (Chapter 2.3.iii).

4.3.2. IBV vaccines

Two different commercial live vaccines were used in this study; i) H120, vaccine belonging to Mass serotype (Merial S.A.S, Lyon, France) and (ii) 793B-type vaccine, strain CR88 (Merial S.A.S, Lyon, France). The vaccines were kept at +4-8°C until used. Each vaccine was dissolved in chilled SDW as follows: H120 vaccine alone, CR88 vaccine alone, or H120 plus CR88 vaccines.

4.3.3. IBV challenge strains

Virulent strains of IBV IS/885 and IS/1494/06-like (referred here as IS/1494) were used as challenge viruses. These viruses had been isolated from a recent outbreak of high mortality and respiratory disease complex in broiler flocks in Egypt (Details source of the viruses described in Chapter 3.3. 1). The IS/885 strain was identified by RT-PCR and sequencing (Chapter 3.3. 1). The IS/1494 strain showed a high sequence 99% nucleotide level similarity to Israel strain IS/1494/06 (GenBank Accesion number: EU780077). The titre of the isolates were determined in SPF ECE, and expressed as 50% EID_{50} (Chapter 2.8.iii).

4.4. Experimental design

Ninety one-day old commercial broiler chicks were divided into three groups of thirty chicks and housed in different isolation rooms. At 1 and 14 days-old, chicks were vaccinated oculonasally with 0.1 ml of vaccine according to the programme described in Table 4.1. Following the vaccinations, the birds were observed daily for clinical signs. Blood was collected prior to vaccination (1 day-old) and at 30 days old from eight chicks in each group for antibody responses. On the same day, ten chicks from each group (vaccinated and control groups) were transferred to another isolation room and challenged by the oculonasal route with 0.1 ml of virulent IS/885 to provide $10^{4.66}$ EID_{50}/chick. Another 10 chicks from each group were similarly transferred and challenged with 0.1 ml of virulent IS/1494 to provide $10^{5.00}$ EID_{50}/chick. The remaining 10 chicks (vaccinated or unvaccinated control) in each group were left as unchallenged controls. The birds were observed daily for clinical signs during the post-challenge period. Five days after challenge, all ten chicks in each unchallenged and challenged group were humanely euthanized (Chapter 2.5.iv). Vaccine protection was evaluated by ciliostasis test, examination of trachea and kidney for gross lesions, and virus detection by RT-PCR.

Table 4.1. Experimental design used to study the protection conferred by two different IBV vaccination programmes against the Middle East isolates of IS/885 and IS/1494.

Group	**Age and vaccination programmes (Oculonasally)**	
	Day old	14 days-old
I	H120	CR88
II	H120+CR88	CR88
III	SW	SW

4.4.1. Detection of antibody responses

Sera collected prior to vaccination (1 day-old) and at 30 days old of age were tested using commercial ELISA (BioChek) following the protocols recommended by the manufacturer (Chapter 2.12.i.a). The HI test was carried out as described in Chapter 2.12.ii.a. The IBV antigens used for the HI assay were M41, 793B, IS/885 and IS/1494 (Chapter 2.12.ii.a).

4.4.2. Ciliostasis test

Tracheas were removed from each chick and processed for ciliary assessment (Chapter 2.5.v). According to the European Pharmacopoeia's, 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).

4.4.3. Gross lesions

All chicks that were sacrificed at 5 day post challenge (dpc) were examined individually for gross tracheal and kidney lesions and scored following the criteria described in Chapter 2.5.ii. Total gross lesions scores for each group were calculated based on the mean of scores observed per total number of chicks.

4.4.3. RT- PCR and DNA Sequencing

Individual tissue samples of trachea and kidney were ground up using a pestle and mortar (Chapter 2.9.iii). RNA was extracted using QIAamp viral RNA mini kit following the manufacturer's instructions (Chapter 2.10.ii). RT-PCR was carried out as described in Chapter 2.10.iii.

Samples positive for IBV were processed for sequencing using EXO-SAP according to manufacturer's instructions and were sequenced using the SX3+ primers (Chapter 2.11).

4.4.4. Statistical analysis

The ELISA, HI antibody titres and gross lesion between groups were analysed statistically using one way ANOVA, followed by Tukey's test for comparison of means. Differences were considered to be significant when $p \leq 0.05$. All analysis were conducted using the GraphPad Prism, 6.0.1.

4.5. Results

4.5. 1. Clinical signs post vaccination

There were no clinical signs found in the unvaccinated control chicks. In both vaccinated groups, mild clinical respiratory signs began to appear at 6 dpv. The signs included head shaking, tracheal râles and sneezes. These signs subsided by 12 dpv.

4.5. 2. Serology

The mean ELISA antibody titre prior to vaccination (1 day-old, MDA) was 4174±225. The means of IBV HI titres against IBV M41, 793B, IS/885 and IS/1494 prior to vaccination were $\log_2$ 6.3, 5.6, 3.5 and 3.1 respectively.

At 30 days of age (on the day of challenge), vaccinated groups showed significantly higher levels of IBV ELISA antibody titre than the unvaccinated control group. Chicks of group II (d0:H120+CR88, d14:CR88) exhibited significantly higher antibody titre ($P<0.05$) than group I (d0:H120, d14:CR88) (Figure 4.1). The HI response to M41 and 793B were higher than the heterologous antigens (IS/885 and IS/1494) (Figure 4.2). Using the M41 as antigen, the vaccinated chickens showed high level of HI antibody titre compared to the control group (Group III). The antibody titre against 793B antigen in group II was significantly higher than group I (Figure 4. 2).

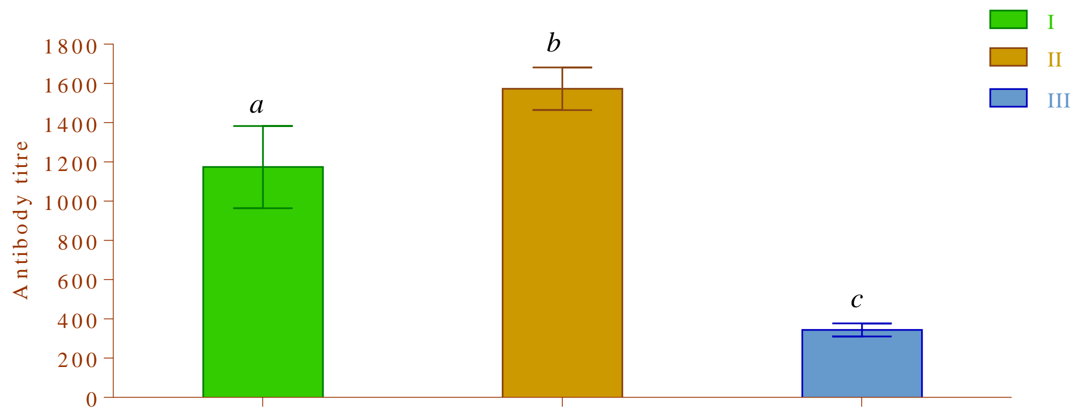

Figure 4.1. Mean ELISA antibody titres at 30 days of age. Group I= d0:H120, d14:CR88; group II= d0:H120+CR88, d14CR88; group; III= control. Bars represent the mean ± SEM (n=8). Different superscripts lowercases letters in the bar indicate significant difference (P<0.05), while data with same letters indicates that there were no significant differences (p > 0.05). The cut-off titre= 834.

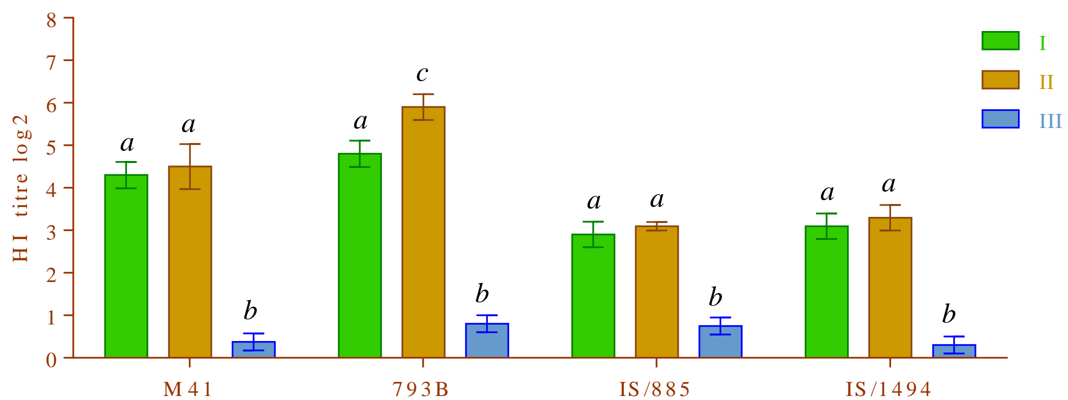

Figure 4.2. HI antibody titres log_2 against IBV antigens at 30 days of age. Group I= d0:H120, d14:CR88; group II= d0:H120+CR88, d14CR88; group III= control. Bars represent the mean ± SEM (n=8). Different superscripts lowercases letters in the bar indicate significant difference (P<0.05), while data with same letters indicates that there were no significant differences (p > 0.05) (within same antigen).

4.5. 3. Clinical signs post challenge

The unvaccinated and vaccinated-unchallenged groups were remained free of clinical signs. All the ten chicks of unvaccinated-challenged chicks showed signs of typical IBV infection at 1 dpc which including depression with ruffled feathers, listlessness and huddling, head shaking, tracheal râles, sneezing and coughing. These signs were continued up to 5 dpc. In contrast, no clinical signs were observed in both of the vaccinated-challenged groups.

4.5. 4. Ciliostasis test

The percentage ciliostasis protection was calculated for each group. The unvaccinated and vaccinated-unchallenged groups had greater than 98% ciliary activity. The unvaccinated (group III) birds challenged with either IS/885 or IS/1494 viruses had protection scores of 0% (Table 4.2). Group II (d0:H120+CR88, d14:CR88) and group I (d0:H120, d14:CR88) showed 83% and 60% ciliary protection against IS/885 challenge respectively (Table 2). Following challenge with IS/1494, the group I and II gave 80% and 94 % protection respectively (Table 4.2).

4.5. 5. Gross lesions

The unvaccinated and vaccinated-unchallenged control groups remained free of gross lesions. Five days after the IS/885 challenge, congestion of the trachea and pale swollen kidneys were observed in all chicks in group III (unvaccinated challenged). These lesions were also found in group I (Table 4.2). However, birds in group II were free of these gross lesions. Following the IS/1494 challenge, congestion of trachea and pale swollen of kidney was observed in one bird in group I, while it appeared normal in the rest of the birds (Table 4.2). Chicks in group II showed no tracheal or kidney lesions.

Table 4.2. Ciliostasis test and gross lesions of IBV-vaccinated and unvaccinated chicks following challenge with IS/885 or IS/1494 isolates.

Groups	IS/885			IS/1494		
	Ciliostasis test	**Gross lesions** *		**Ciliostasis test**	**Gross lesions**	
	% Protection scores	Trachea	Kidney	% Protection scores	Trachea	Kidney
I	60	0.3±0.1 [a]	0.6±0.1 [b]	80	0.1±0.1 [a]	0.1±0.1 [a]
II	83	0.0±0.0 [a]	0.0±0.0 [a]	94	0.0±0.0 [a]	0.0±0.0 [a]
III	0	0.9±0.1 [b]	0.9±0.1 [b]	0	0.8±0.1 [b]	0.8±0.1 [b]

*Group I= d0:H120, d14:CR88; group II= d0:H120+CR88, d14CR88; group III= unvaccinated control. * Severity of gross lesions induced by virulent IBV infection and data are expressed as the mean gross lesion score ± SEM (n=10). Different superscripts lowercases letters within same columns indicate significant difference ($P<0.05$), while data with same letters indicates that there were no significant differences ($p > 0.05$).*

4.5. 6. Post challenge virus detection

The number of birds positive for detection of IBV genome in the trachea or kidney, five days after the virulent IS/885 or IS/1494 challenges, are presented in the Table 4.3 and Table 4.4 respectively. In the unvaccinated-challenged groups, either challenged by IS/885 or IS/1494, the challenge virus was detected in the trachea and kidney of all 10 chicks. Following the IS/885 challenge, for group I (d0:H120; d14:CR88), 9 tracheas and two kidneys were positive for the challenge virus. In contrast, in group II, no challenge virus was detected in kidney but four tracheas were positive for IBV (two vaccinal and the other two challenge viruses). When the groups were challenged with IS/1494, in group I, two of 10 and 6 of 10 kidney and trachea respectively were positive for the challenge virus. For the trachea, one of 10 tracheas was positive for the CR88 and the rest were negative for viruses. In group II, no virus was detected in trachea but one of 10 kidneys was positive for CR88.

Table 4.3. Detection of the vaccine or challenge virus of IS/885 by RT-PCR in the trachea and kidney at 5 dpc.

Groups	Tissue samples	Number of birds									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
I	Trachea	Ch*	Ch	Ch	-	Ch	Ch	Ch	Ch	Ch	Ch
	Kidney	-	-	-	-	Ch	Ch	-	-	-	-
II	Trachea	CR88	-	CR88	-	Ch	-	-	Ch	-	-
	Kidney	-	-	-	-	-	-	-	-	-	-
III	Trachea	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch
	Kidney	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch

**Challenge virus (99 to100% part-S1 sequence similarity to the initial inoculum of IS/885). Group I= d0:H120, d14:CR88; group II= d0:H120+CR88, d14CR88; group III= unvaccinated control.*

Table 4.4. Detection of the vaccines or challenge virus of IS/1494 by RT-PCR in the trachea and kidney at 5 dpc.

Groups	Tissue samples	Number of birds									
		1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
I	Trachea	-	Ch*	Ch	-	-	Ch	Ch	Ch	CR88	Ch
	Kidney	-	-	-	-	Ch	-	-	-	-	Ch
II	Trachea	-	-	-	-	-	-	-	-	-	-
	Kidney	-	CR88	-	-	-	-	-	-	-	-
III	Trachea	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch
	Kidney	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch	Ch

* *Challenge virus (99 to100% part-S1 sequence similarity to the initial inoculum of IS/1494). Group I= d0:H120, d14:CR88; group II= d0:H120+CR88, d14CR88; group III= unvaccinated control.*

4.6. Discussion

The evaluation of protection conferred by live vaccines against the virulent IS/885 and IS/1494 isolates was assessed based on ciliary activity in the tracheal explants prepared from vaccinated-challenged chicks (Darbyshire, 1980; Marquardt et al., 1982; Andrade et al., 1982; Snyder et al., 1983; Cook et al., 1999a), gross lesions and virus detection in the trachea and kidneys following the challenge with the respective viruses. The combined vaccination programme where both live H120 and CR88 vaccines were simultaneously given at one day-old followed by CR88 vaccine two weeks later (Group II) provided an excellent protection against both isolates. Following the challenges, there were no clinical signs or tracheal/kidney lesions and the ciliary protection was high (83% to 94%). While 100% of the unvaccinated challenged birds were positive for IBV RNA in the trachea and kidney, in group II, no RNA was detected in kidneys and only 20% of the trachea was positive for the challenge virus.

Live H120 vaccine alone at day old followed by CR88 vaccine two weeks later (group I) conferred somewhat poorer protection against IS/885 than group II. Protection was much improved when the CR88 was given together with the H120 at day-old in contrast to H120 given alone. It must be noted that the vaccine programme given to group II also offered 100% kidney protection against both isolates. In a previous study performed in SPF chicks, live H120 vaccination afforded protection of the trachea (92%) and the kidney (25%) on the basis of virus isolation when the birds were challenged with IS/885/00 (Meir et al., 2004). Moreover, immunization with live H120, H52, and D274 given singly was reported to produce little cross protection against challenge with other nephropathogenic IBV strains (Lambrechts et al., 1993; Pensaert and Lambrechts, 1994). Our study shows

that the vaccination programme of group II has further boosted the ciliary and tracheal/kidney protection against IS/885 compared to that of group I.

Both vaccination programmes used in this experiment provided excellent protection against the virulent IS/1494 challenge. A better protection observed in group II (d0:H120+CR88, d14CR88) as compared to group I (d0:H120, d14CR88), could be due to the higher levels of local and cellular immunity at the tracheal site which may have prevented the virulent virus from reaching the kidneys (Lambrechts et al., 1993). In an another experiment, cellular and local immunity induced by administration of combined live Massachusetts and 793B-type vaccines at day old showed a significant increase in the expression of CD4+, CD8+ and IgA-bearing B cells in the trachea compared to single H120 alone or unvaccinated groups (Chapter 5). These results reinforce the importance of optimising local and cell-mediated mucosal immunity at the respiratory lining through strategic heterologous day-old vaccination for enhanced protection against variant viruses.

As the challenge virus could be differentiated from the vaccinal strains by part-S1 sequencing in this study, the trachea and kidney were examined for the presence of the respective challenge viruses following the challenge. Upon excluding birds with vaccine virus detection, group II displayed 80% or more tracheal protection and no virus (100% protection) in the kidney, following the IS/885 or IS/494 challenges. This demonstrates excellent protection by this vaccination programme and this supports the other findings (eg. clinical signs, gross lesions and ciliary scores). In contrast, group I showed poorer protection, particularly in the trachea. While the tracheal protection was only 10% and 40% against IS/885 and IS/1494 challenge viruses respectively, the kidney of 80% of the birds were protected. This shows that vaccination programme in group 1 (d0:H120; d14:CR88) provides lower protection

against these challenge viruses, particularly against IS/885. In some cases, where the challenge virus can be differentiated from vaccinal IBV strains, redetection of the challenge virus in trachea and kidney can be used as part of protection studies.

Humoral antibody responses following vaccination as measured by ELISA and HI are often used for monitoring vaccine-take (Dhinakar Raj and Jones, 1997). In this study, at 30 days of age (challenge day) the mean ELISA titre and the mean 793B HI titre in group II were significantly higher than the other vaccinated group. This reflects that combined live IBV vaccination at day-old and re-vaccination at 14 days-old has provided an immunological boost compare to the other vaccination schedule (group I). Although it has been demonstrated before that circulating humoral antibody levels are of minor importance in the protection against IBV infection (Raggi and Lee, 1965; Darbyshire and Peters, 1985; Endo-Munoz and Faragher, 1989; Pensaert and Lambrechts, 1994), in our study the group with higher levels of humoral antibodies had better protection against both of the Middle East isolates. Cytotoxic T cell plays a vital anti-viral activity during early stages of IBV infection while serum IgG is critical at the late stage of IBV infection (Collisson et al., 2000).

Despite the low level of genetic homology in the S1 protein between the vaccine and challenge strains used in this study, the protection achieved with the vaccination programme employed in the present study was high. The genetic relationship of the hypervariable region of the S1 gene between IS/885 and H120 or CR88 was 80% and 79% respectively, while the genetic relationship between the IS/1494 and H120 or CR88 was 81% and 80% respectively. Despite the 19-21% variation in the S1 gene, the successful protection conferred by vaccination programmes employed in

this study may be attributable to the shared characteristics of the S1 protein of the vaccine and the challenge IBVs (Cavanagh et al., 1986a).

Based on the data presented in this study, it appears that a combination of live H120 and CR88 vaccines given at day old followed by CR88 vaccine at day 14 of age confer an excellent protection against virulent variant IS/885 and IS/1494 viruses. More work is needed to establish the underlying immune mechanisms for such higher and broader protection conferred by these distant vaccine viruses.

Chapter 5: Effects of homologous and heterologous live IBV vaccination in day-old commercial broiler chicks: tracheal health, immune responses and protection

5.1. Abstract

Group of commercial broiler chicks were vaccinated at one day-old with seven live IBV vaccines which included two different Mass-type and two different 793B-type vaccines. Five chicks were humanly killed at intervals and tracheal samples were collected for ciliary activity assessment and for the detection of CD4+, CD8+ and IgA-bearing B cells by IHC. Blood samples were collected regularly for the detection of IBV antibody responses by ELISA. Protection studies were conducted to evaluate immunity conferred by different vaccination regimes against virulent IBV M41, QX and 793B strain challenges. Chicks receiving two vaccines showed substantial damage to the trachea epithelium at 10 and 14 dpv compared to those given single vaccines. All vaccine viruses were able to induce measurable levels of CD4+, CD8+ and IgA-bearing B cells in the trachea following vaccination when compared to unvaccinated birds. At peak time, significantly higher levels of CD4+ (6 dpv), CD8+ (14 dpv) expression were observed in the dual vaccinated groups when compared to single vaccine groups. These results indicate variations in the development of CMI following live IBV vaccinations at day-old. At the time of challenge there were low levels or no circulating antibodies against IBV detected by ELISA. Protection studies indicated that the group of chicks vaccinated with $Mass_2$+$793B_2$ produced good cross protection against challenge with IBV QX when compared to $Mass_1$+D274 or $793B_1$. All vaccines produced solid immunity (>80% protection) against homologous challenge from IBV M41 and 793B.

5.2. Introduction

Infectious bronchitis is controlled by the administration of live, IBV attenuated vaccines and it has been suggested that mucosal immunity is essential for effective protection against the virus (Gomez and Raggi, 1974). The development of local immunity may rely on the direct interaction between mucosal immune system elements and IBV itself (Toro et al., 1997; Guo et al., 2008). Previous studies have reported the development of humoral immune responses following live IBV vaccination (Cook et al., 1999a; Terregino et al., 2008). However, conflicting studies demonstrate that humoral responses have a low correlation with protection against IBV infection (Raggi and Lee, 1965; Roh et al., 2013). Instead, they highlighted the importance of the local and CMI in IBV infections.

Trachea being a main target of IBV, cellular and local immunity in that organ has been demonstrated (Nakamura et al., 1991; Dhinakar Raj and Jones, 1996a; Kotani et al., 2000). It has been shown that IgA and CD8+ T cell responses which develop at tracheal sites following IBV vaccination could be taken as a good indicator of protection against the virus (Okino et al., 2013). Local anti-IBV antibodies, particularly IgA and cytotoxic T cells, have been shown as crucial for restricting or eliminating IBV from the host (Gillette, 1981; Collisson et al., 2000; Mondal and Naqi, 2001). It has been demonstrated that lachrymal fluid IgA levels of chickens are associated with resistance against IBV infection (Toro and Fernandez, 1994). However, little information is available regarding the evaluation of cellular and local immune responses elicited by different live IBV vaccination programmes.

It well recognized that IBV consists of more than one serotype. Mass 41 and 793B serotypes have spread worldwide and commercial vaccines are available (Bijlenga et

al., 2004; Jones, 2010). Despite vaccination, different and novel serotypes continue to be a major concern in poultry of all ages in many parts of the world (de Wit et al., 2011a). In addition, the continuous emergence of novel antigenic variants makes prevention of IBV infections challenging. The QX serotype was first isolated in China in 1996 from birds with proventriculitis (Wang et al., 1998), and later reported in Europe (Beato et al., 2005; Worthington et al., 2008), Middle East (Amin et al., 2012), Africa (Toffan et al., 2011a), and rapidly spread to become the most widespread serotype of non-vaccine origin (Worthington et al., 2008).

To achieve broad protection against challenge from IBV variants, the use of combinations of different live IBV vaccines has been shown to induce a wide of protection against challenge from several heterologous strains (Cook et al., 1999a; Gelb et al., 2005). The phenomenon of IBV cross-protection has been recognized for many years (Hofstad, 1981; Cook et al., 1999a), and has been attributed to the host immune response towards several IBV gene epitopes, especially the S1 sub-unit (Cavanagh et al., 1997).

This study aimed to evaluate the tracheal health of chicks following vaccination with a number IBV strains belonging to serotypes Mass, D274 and 793B, or Ark using ciliary activity assessment. The cellular and local immune responses in trachea were assessed using IHC. The humoral antibody responses against IBV were also evaluated using a commercial ELISA kit. Furthermore, the protection conferred by different vaccine programmes against virulent IBV M41, QX and 793B was evaluated. Following challenge, clinical signs, ciliary activity and virus detection by RT-PCR were carried out.

5.3. Materials and methods

5.3.1. Chicks

Day-old commercial broiler chicks with IBV MDA were obtained from a commercial hatchery (Chapter 2.3.ii).

5.3.2. IBV vaccines

Seven commercially available live IBV vaccines were used. They belong to four different serotypes, namely Mass, 793B, D274 and Ark. Vaccines '$Mass_1$' and '$Mass_2$' contained two different strains of the Mass serotypes. Two commonly used 793B vaccines, referred here as $793B_1$ and $793B_2$ were used in combination with the Mass vaccines. The mixtures of $Mass_1$+$793B_1$ and $Mass_2$+$793B_2$ were prepared in our laboratory. Vaccine '$Mass_1$+D274' is a commercial combined live vaccine containing both a Mass and D274-type strain. Vaccine '$Mass_3$+Ark' contained a manufactured combination of a Mass and Ark-type strain. All vaccines were prepared prior administration and to provide the dosages per chick as recommended by the manufacturers.

5.3.3. IBV challenge strains

Virulent IBV challenge viruses belonging to three different serotypes were used. M41 has been maintained in our laboratory for several years (Dhinakar Raj and Jones, 1996b). QX (KG3P) strain was first isolated from the proventriculus of a flock of broilers in England (Ganapathy et al., 2012). The 793B (KG12/11) strain was isolated from caecal tonsil that came from a flock of layers suffering from a drop in egg production (Ganapathy, unpublished data). All viruses were grown in ECE and titrated in TOC as described in Chapter 2.8.

5.4. Experimental design

Three hundred and eighty five, day-old chicks were randomly divided into seven groups and kept in separate isolation units, with 55 chicks per group. Each group was inoculated with one of the following, $Mass_1$ (group 1), $Mass_2$ (group 2), $Mass_1$+D274 (group 3), $Mass_1$+$793B_1$ (group 4), $Mass_2$+$793B_2$ (group 5), $Mass_3$+Ark (group 6) and SW (group 7, control). Each chick was inoculated via oculo (50 µl) and nasal (50 µl) routes. Dosages were given as recommended by the manufacturer. Following vaccination, chicks were observed daily for clinical signs as described in Chapter 2.5.i.

5.4.1. Study 1. Evaluation of tracheal health of chicks following live IBV vaccinations

At 3, 6, 10, 14, 18 and 25 dpv, five chicks from each group were humanely killed. Tracheas were removed from each chick and processed for ciliostasis test as described in Chapter 2.5.v.

5.4.2. Study 2. Evaluation of tracheal immunity induced by live IBV vaccines

During necropsy, pieces of the trachea were collected at 3, 6, 10, 14, 18 and 25 dpv from five chicks in each group. Pieces of trachea were used for the detection of CD4+, CD8+ and IgA-bearing B cells by IHC as described in Chapter 2.13.

5.4.3. Study 3. Evaluation of humoral antibody response induced by live IBV vaccines

IBV antibodies were detected using a commercial ELISA kit (BioChek) following the protocols recommended by the manufacturer (Chapter 2.12.i.a). Serum was collected prior to vaccination and then at 3, 6, 10, 14, 18 and 25 dpv from eight chicks per group to evaluate the antibody response against IBV.

5.4.4. Study 4. Assessment of protection induced by homologous or heterologous vaccination against virulent IBVs

At 21 days of age, five chicks were taken from each group and challenged oculonasally with $10^{5.00}$ CD_{50} M41 per dose of 0.1 ml. The same number from each group was challenged with $10^{5.00}$ CD_{50}/ml QX per dose of 0.1 ml, and a further five birds from the combined vaccine groups were challenged with $10^{4.50}$ CD_{50}/ml 793B per dose of 0.1 ml via the same route. The remaining chicks in each group were left unchallenged in control groups. Following challenge, all birds were observed daily for clinical signs attributable to IBV infection (Chapter 2.5.i). Five dpc, the ciliary activity (Chapter 2.5.v) of tracheal explants was examined in both the challenged and unchallenged chicks. To evaluate the effect of vaccines on the prevention of shedding of the challenge virus, trachea and kidney tissue samples were collected and processed for virus detection by RT-PCR (Chapter 2.10). PCR positive products

corresponding to the S1 were purified with EXO-SAP for partial DNA sequencing (Chapter 2.11).

5.5. Statistical analysis

Statistical analysis of cellular, local and humoral antibody responses data was conducted using one way ANOVA, followed by Tukey's test. Differences were considered to be significant when $p \leq 0.05$. All analysis was conducted using GraphPad Prism, 6.0.1.

5.6. Results

5.6.1. Clinical signs following vaccination

A summary of the onset and duration of the clinical signs following vaccination for each vaccine group is shown in Figure 5.1. Control birds (group 7) remained free of clinical signs throughout the experiment. For groups that received single Mass strain vaccines (group 1 or 2), mild respiratory clinical signs of short duration such as head shaking, tracheal râles, sneezing and coughing appeared from 6 dpv and continued until 10 dpv. Groups 3 and 4 that were vaccinated with $Mass_1$ combined with either D274 or $793B_1$ showed mild clinical signs starting at 5 dpv and subsiding by 14 dpv. Group 5 ($Mass_2$+$793B_2$), mild clinical signs were first observed at 2dpv, starting with coughing and sneezing. At 4 dpv some of the chicks were showing depression, ruffled feathers, coughing, gasping and up to 10 dpv, but thereafter the chicks showed mild respiratory signs and ultimately all signs disappeared at 14dpv. For group 6 ($Mass_3$+Ark), signs of mild respiratory distress started at 2 dpv and continued up to 10 dpv.

Despite showing only mild clinical signs, two birds in group 1 were dead at 6 and 8 dpv, one bird died in group 2 at 2 dpv, two birds died in group 3 at 8 and 14 dpv and one bird died in group 4 at 14 dpv. No gross lesions were observed during post mortem examination. In group 5, during the stage when severe clinical signs were seen, three birds died at 6, 9 and 10 dpv. Post-mortem examination showed fibrinous pericarditis, peritonitis and perihepatitis in all birds. No deaths were recorded in group 6 or 7 (control).

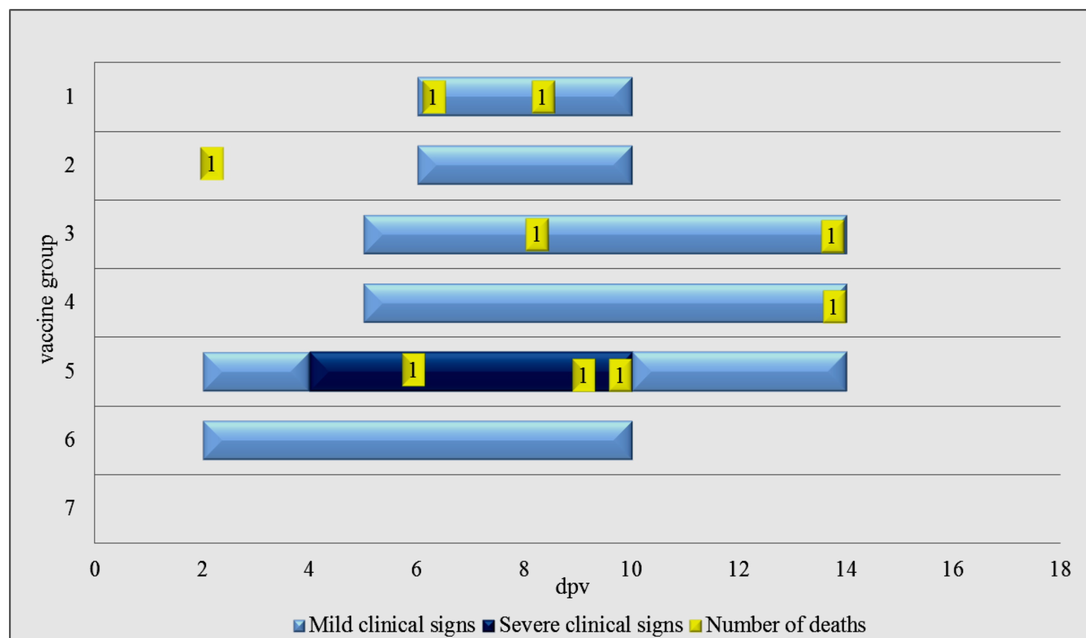

Figure 5.1. Onset and duration of clinical signs and mortality within each of the seven vaccine groups. Group 1 = $Mass_1$, Group 2 = $Mass_2$, Group 3 = $Mass_1$+D274, Group 4 = $Mass_1$+$793B_1$, Group 5 = $Mass_2$+$793B_2$, Group 6 = $Mass_3$+Ark and Group 7 = SW.

5.6.2. Study 1. Tracheal health of chicks following live IBV vaccinations

Results of the ciliary activity assessment are presented in Figure 5.2. Chicks vaccinated with a single live IBV vaccine (groups 1 and 2) showed no effect on trachea epithelium activity when compared to the unvaccinated birds. Between dually-vaccinated groups, the pattern of damage to the ciliary activities differed. Severe damage to the tracheal epithelium was seen in both groups 3 and 4 at 10 and 14 dpv respectively compared to group 1 vaccinated solely with $Mass_1$. Similarly, groups 5 and 6 demonstrated severe damage at 10 dpv when compared to group 2. Tracheal epithelium cells of the vaccinated groups were fully (≥ 80% protection) recovered at 18-25 dpv.

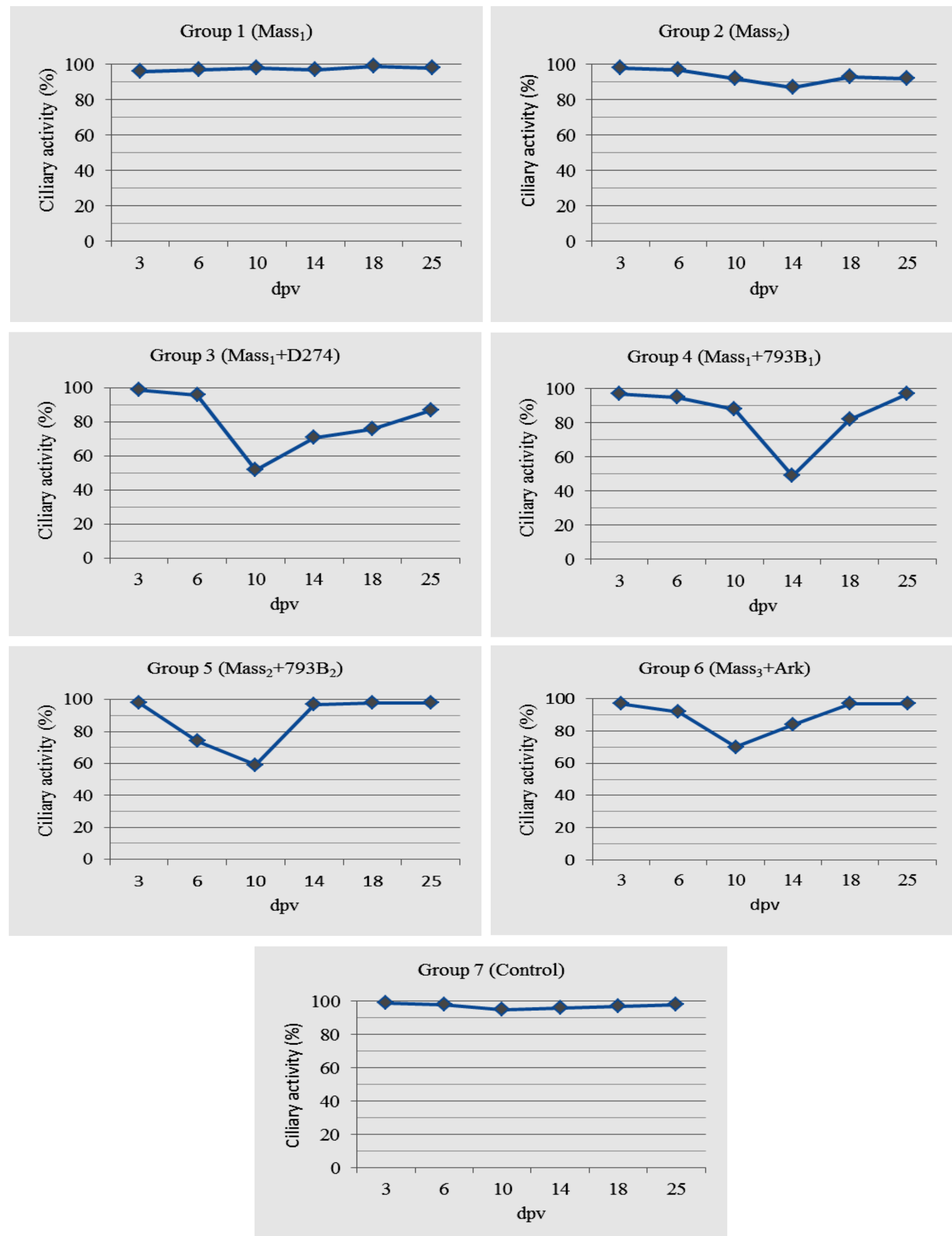

Figure 5.2. Comparison of ciliary activity in chicks receiving either one or two vaccines. Group 1 = $Mass_1$, Group 2 = $Mass_2$, Group 3 = $Mass_1$+D274, Group 4 = $Mass_1$+$793B_1$, Group 5 = $Mass_2$+$793B_2$, Group 6 = $Mass_3$+Ark and Group 7 = SW.

5.6.3. Study 2. Tracheal immunity induced by live IBV vaccinations

All vaccine viruses induced measurable levels of CD4+, CD8+ (Table 5.1) and IgA-bearing B (Table 5.2) cells in the trachea following vaccination when compared to unvaccinated birds. CD4+ and CD8+ cell counts varied between each vaccinated group throughout sampling time. However, in all vaccinated groups the expression levels of CD4+ increased from 3 dpv, peaked at day 6 and then decreased dramatically after 10 dpv. One exception was a significantly high expression ($p < 0.05$) of CD4+ cells at 3 dpv for groups 2, 5 and 6. At 6 dpv a significantly high level of CD4+ cells was observed in the dual vaccinated groups (group 3, 4, 5 and 6) when compared to the single vaccine groups (group 1 and 2). At 10 dpv, group 5 and 6 showed a significantly high level of CD4+ compared to all other vaccinated groups. However levels diminished by 25 dpv and no significant differences were seen between vaccinated and control groups.

For CD8+ cell counts, no significant differences were observed at 3 dpv in vaccinated groups when compared to the control group. The average number CD8+ cells increased after 6 dpv and peaked by 14-18 dpv. At 10 dpv, high significantly levels of CD8+ were observed in combined vaccine groups (group 3, 4, 5 and 6) when compared to the single vaccine groups (group 1 and 2). By 14 dpv, no significant differences were observed between any of the vaccinated groups. The average number of CD8+ cells subsided by 25 dpv. The decline of CD4+ cells corresponded with an increase of CD8+ cells.

All vaccinated groups demonstrated a significantly higher IgA-bearing B cell count when compared to the unvaccinated group throughout sampling time. Local IgA-bearing B cells levels peaked at 10 dpv, and in addition, the level of

IgA-bearing B cells was significantly higher ($p < 0.05$) in dual vaccinated groups (group 3, 4, 5 and 6) than single vaccinated groups (group 1 and 2).

Table 5.1. Immunohistochemical detection of CD4+ and CD8+ cells in the trachea of chickens receiving IBV vaccinations.

Vaccine Groups	CD4+ dpv 3	6	10	14	18	25	CD8+ dpv 3	6	10	14	18	25
1	36 ± 0.3^{a}	46 ± 4.8^{a}	52 ± 4.3^{a}	37 ± 4.3^{ab}	17 ± 0.8^{a}	12 ± 0.3	11 ± 0.1	22 ± 1.7^{a}	38 ± 11^{ab}	53 ± 7.4^{a}	66 ± 0.6^{a}	31 ± 0.7
2	71 ± 0.4^{b}	48 ± 2.8^{a}	34 ± 4.7^{ac}	11 ± 1.3^{a}	22 ± 0.5^{a}	9.0 ± 0.2	14 ± 0.3	24 ± 4.9^{a}	27 ± 5.1^{a}	54 ± 4.4^{a}	48 ± 14^{a}	14 ± 0.3
3	35 ± 0.3^{a}	79 ± 8.7^{b}	34 ± 3.7^{c}	58 ± 0.6^{b}	37 ± 10^{ab}	13 ± 0.2	16 ± 4.5	$14.\pm1.3^{ab}$	43 ± 7.1^{b}	80 ± 10^{a}	51 ± 0.3^{a}	21 ± 12
4	50 ± 4.5^{a}	70 ± 5.1^{b}	29 ± 3.7^{c}	44 ± 0.2^{ab}	52 ± 16^{b}	25 ± 0.6	25 ± 0.6	21 ± 0.2^{a}	49 ± 7.1^{ab}	57 ± 0.4^{a}	59 ± 0.4^{a}	25 ± 11
5	79 ± 0.6^{b}	93 ± 7.5^{b}	81 ± 0.6^{b}	35 ± 0.3^{ab}	17 ± 0.3^{a}	15 ± 0.4	24 ± 0.8	19 ± 2.4^{ab}	87 ± 18^{b}	77 ± 2.9^{a}	58 ± 2.7^{a}	18 ± 0.2
6	76 ± 0.6^{b}	70 ± 0.7^{b}	62 ± 5.6^{b}	68 ± 21^{b}	27 ± 0.7^{a}	14 ± 0.3	28 ± 10	29 ± 2.4^{a}	12 ± 0.7^{a}	68 ± 21^{a}	56 ± 0.6^{a}	12 ± 0.7
7	8 ± 0.1^{c}	13 ± 0.2^{c}	10 ± 0.1^{d}	6.0 ± 0.5^{a}	8.0 ± 0.2^{a}	4.0 ± 0.1	6.0 ± 0.3	9.2 ± 0.2^{b}	7.0 ± 0.1^{a}	5.6 ± 0.6^{b}	8.2 ± 0.2^{b}	12 ± 0.1

Data is expressed as mean values ± SEM. Significant differences within each column (dpv) are labelled with either a, b or ab. Groups with no significant difference between them are labelled with the same letter, whereas groups with a significant difference are labelled with a different letter ($p < 0.05$). Time points with no significant differences are not labelled. Group 1 = Mass$_1$, Group 2 = Mass$_2$, Group 3 = Mass$_1$+D274, Group 4 = Mass$_1$+793B$_1$, Group 5 = Mass$_2$+793B$_2$, Group 6 = Mass$_3$+Ark and Group 7 = SW.

Table 5.2. Immunohistochemical detection of IgA-bearing B cells in the trachea of chickens receiving IBV vaccinations.

Vaccine Groups	dpv					
	3	**6**	**10**	**14**	**18***	**25***
1	29±0.6[a]	40±0.6[a]	70±0.4[a]	66±0.4[a]		
2	31±11[a]	60±12[a]	84±0.9[ab]	77±0.5[ab]		
3	58±0.9[a]	50±0.9[a]	72±21[a]	59±20[a]		
4	52±0.8[a]	60±10[a]	96±15[ab]	70±13[ab]		
5	40±13[a]	68±19[a]	122±15[b]	102±22[b]		
6	42±14[a]	70±15[a]	88±14[ab]	78±0.5[ab]		
7	5.0±0.1[b]	4.0±0.5[b]	10±0.4[c]	10±0.4[c]		

[*]No data were obtained for 18 or 25 dpv. Data is expressed as mean values ± SEM. Significant differences within each column (dpv) are labelled with either a, b or ab. Groups with no significant difference between them are labelled with the same letter, whereas groups with a significant difference are labelled with a different letter ($p < 0.05$). Time points with no significant differences are not labelled. Group 1 = $Mass_1$, Group 2 = $Mass_2$, Group 3 = $Mass_1$+D274, Group 4 = $Mass_1+793B_1$, Group 5 = $Mass_2+793B_2$, Group 6 = $Mass_3$+Ark and Group 7 = SW.

5.6.4. Study 3. Humoral antibody response induced by live IBV vaccinations

The mean ELISA antibody titre in the chicks at day-old was 5702±324. Mean titres of each group following vaccination are shown in Table 5.3. At 3 dpv, MDA levels began to decline in all groups, although group 5 ($Mass_2$+$793B_2$) had a significant reduction in antibody titre ($p < 0.05$) when compared to the other groups. At 6 dpv, no significant differences were seen between the vaccinated and control groups. By 10 dpv, all groups had no detectable antibody titres, except for group 6 ($Mass_3$+Ark) which was above the detectable titre level (Table 5.3). Despite an increase in antibody titres in four vaccinated groups at 18 dpv, all groups remained below detectable levels until end of the experiment.

Table 5.3. Mean ELISA antibody titres for each group receiving either single or combined vaccinations.

Group	dpv					
	3	**6**	**10**	**14**	**18**	**25**
1	3463±583[a]	1421±236	570±78[ab]	205±30[a]	401±57	314±57[ab]
2	2466±380[a]	2128±314	675±75[ab]	385±60[ab]	209±45	431±93[ab]
3	2324±254[a]	2108±380	440±94[a]	328±77[ab]	406±72	391±77[ab]
4	2374±334[a]	1788±355	748±101[ab]	291±41[ab]	404±178	639±97[a]
5	1846±199[b]	1911±208	501±49[ab]	216±47[ab]	238±56	512±47[a]
6	3094±379[a]	2049±205	881±75[b]	578±96[b]	461±57	444±41[a]
7	2253±392[a]	1728±183	695±107[ab]	290±28[ab]	241±29	128±21[b]

Data is expressed as mean values ± SEM. Significant differences within each column (dpv) are labelled with either a, b or ab. Groups with no significant difference between them are labelled with the same letter, whereas groups with a significant titre difference are labelled with a different letter ($p < 0.05$). Time points with no significant differences are not labelled. Cut-off point titre = 834. Group 1 = $Mass_1$, Group 2 = $Mass_2$, Group 3 = $Mass_1$+D274, Group 4 = $Mass_1$+$793B_1$, Group 5 = $Mass_2$+$793B_2$, Group 6 = $Mass_3$+Ark and Group 7 = SW.

5.6.5. Study 4. Protection induced by homologous or heterologous vaccination against virulent IBVs

Following challenge with virulent M41, QX and 793B strains, clinical signs such as head shacking, sneezing, tracheal râles and coughing were observed in the unvaccinated control group. No clinical signs were observed in vaccinated birds.

Challenge by M41, QX or 793B caused severe damage to the tracheal epithelium of unvaccinated birds (Table 5.4). The results of the ciliary scores show that all the vaccination programmes gave excellent protection against challenge with homologous M41. Group 5 ($Mass_2$+$793B_2$) was the only group protected against challenge from QX, whereas the rest of the groups provided poor protection against challenge.

Only groups receiving a combined vaccine (groups 3, 4, 5 and 6) were challenged with IBV 793B, with all combined vaccines providing good levels of protection (>85% protection). All non-challenged groups retained 100% ciliary beating.

Table 5.4. Homologous and heterologous protection induced by IBV vaccination programmes against virulent IBV challenges at 21 dpv.

Vaccine Group	% Protection scores (ciliostasis test)		
	M41	**QX**	**793B**
1	88	54	ND
2	94	42	ND
3	90	53	90
4	96	68	93
5	90	92	93
6	98	73	85
7	0	15	0

ND= Challenge not done. Group 1 = $Mass_1$, Group 2 = $Mass_2$, Group 3 = $Mass_1$+D274, Group 4 = $Mass_1$+$793B_1$, Group 5 = $Mass_2$+$793B_2$, Group 6 = $Mass_3$+Ark and Group 7 = SW.

5.6.5.1. RT-PCR detection and genotyping of IBVs in trachea and kidney following virulent IBV challenge

The viral genome of each challenge virus was detected in both the trachea and kidney of the control chicks at 5 dpc (Table 5.5). Virulent M41 and 793B were not detected by RT-PCR in any of the vaccinated groups, however the viral genome of the virulent QX was detected in four groups (groups 4 and 5 were negative).

Table 5.5. IBV detection by RT-PCR at 5 dpc in each vaccine group following challenge at 21 dpv.

Vaccine Group	M41		QX		793B	
	Trachea	Kidney	Trachea	Kidney	Trachea	Kidney
1	-	-	QX*	QX*	ND**	ND**
2	-	-	QX*	$Mass_2$	ND**	ND**
3	$Mass_1$	-	QX*	QX*	-	-
4	-	$793B_1$	$Mass_1$	$793B_1$	$793B_1$	$Mass_1$
5	-	$793B_2$	$793B_2$	-	-	-
6	-	-	QX*	-	-	Ark
7	M41*	M41*	QX*	QX*	793B*	793B*

**Challenge strain; **ND = Challenge not done*. Group 1 = $Mass_1$, Group 2 = $Mass_2$, Group 3 = $Mass_1$+D274, Group 4 = $Mass_1$+$793B_1$, Group 5 = $Mass_2$+$793B_2$, Group 6 = $Mass_3$+Ark and Group 7 = SW.

5.7. Discussion

The ciliostasis test was used in the initial study to determine the effect of administration of live IBV vaccines alone or in combination. To our knowledge this is the first report on such findings over an extended period. Our findings show that application of single Mass vaccination causes little detrimental effect on the tracheal ciliary activity compared to that caused by the combined vaccination. One possible explanation is that the combined vaccines contained a higher overall dose than the single vaccines, which may be the cause of the greater damage, because IBV tends to localize subepithelially after initial infection of the surface tracheal epithelial cells (Geilhausen et al., 1973). It has been previously shown that exposure to a higher titre of live attenuated virus results in mild inflammation of the trachea (Gillette, 1981).

Between the combined vaccination groups, the pattern of damage to the ciliary activities differed. For example, group 4 ($Mass_1$+$793B_1$), the tracheal protection fell within 10 days with later recovery compared to group 5 ($Mass_2$+$793B_2$) where the ciliary protection was lowest at first sampling (3 dpv) following vaccination and but showed a slow recovery thereafter. Even though $Mass_1$ and $Mass_2$ or $793B_1$ and $793B_2$ vaccines belong to the Mass or 793B serotypes respectively, when they were used in combination, they showed a high variation on their effect on the tracheal health. Differences in the virulence of IBV strains may have played a role in the tracheal damage. It has been found that unvaccinated chicks challenged with four IBV isolates (one belonging to the Mass serotype and three variants) the tracheal damage in term of ciliary activity was variable (Cubillos et al., 1991). The severity of the ciliostasis caused by virulent 793B strain proved to be the mild, while the effect of M41 was more severe (Benyeda et al., 2009). The results from this study

suggest that such differential effects of different vaccine or vaccination regimes on tracheal health should be utilized in designing proper vaccination programmes.

Relatively little research exists regarding cellular and local immune responses induced by IBV vaccination. To further our understanding, we evaluated CD4+, CD8+ and IgA-bearing B cell expression in the trachea following vaccination regimes used in this study. It is known that presence of CD4+ and CD8+ cells in large numbers in IBV vaccinated or infected birds have a protective role against viral infections (Kotani et al., 2000). Our study reported detection of both types of T cells as early as 3 dpv, which then peaked at 6 dpv (CD4+) and 14 dpv (CD8+). These findings are similar to those by Kotani *et al*. (2000) who identified that CD4+ and CD8+ cell numbers peaked at five days following infection with virulent IBV. Moreover, the present study revealed that CD4+ cells were recruited into the trachea earlier than CD8+. This observation was in accordance with a previous study using a nephropathogenic IBV strain, which observed that on day 5 post infection, CD4+ cells outnumbered CD8+ cell (Janse et al., 1994). In contrast, Dhinakar Raj and Jones (1996a) reported that CD8+ cells were recruited into the trachea earlier than CD4+ cells after infection with virulent 793B. Whether this discrepancy relates to the virulence of the different IBV strains requires further investigation, as attenuated live IBV vaccines were used in the current study.

A greater expression in the levels of CD4+ (6dpv) and CD8+ (10 dpv) were observed in groups receiving the combined vaccines compared to the single vaccines. It is possible that local inflammation and trachea damage caused by a greater vaccination dosage or heterogeneity of vaccines promoted an enhanced response in the respiratory tract. Further to this, a stronger cellular immunity was observed in the group given $Mass_2$+$793B_2$ (group 5) and $Mass_3$+Ark (group 6).

Nakamura et al. (1991) observed IgA cells in the trachea from 7-12 days following infection with virulent IBV M41. We observed in all vaccinated groups that IgA-bearing B cells in the trachea appeared at 3 dpv and peaked at 10 dpv. In our study, the highest level of IgA-bearing B cells was observed in the group given $Mass_2$+$793B_2$. It suggests that an increase in the number of this cells at the tracheal site as a result of greater tracheal damage (Nakamura et al., 1991).

Cellular and local immunity was generated at an earlier stage of infection (3-10 dpv) than humoral immunity (25dpv and thereafter). This is in agreement with previous work that alluded to cytotoxic T cells acting as a vital component for anti-viral activity during the early stage of IBV infection, whereas serum IgG was critical at a later stage (Seo and Collisson, 1997; Collisson et al., 2000).

Following live IBV vaccination, no significant increases in serum antibody titres were found. It is well documented that low or undetectable antibody titres in young chicks following IBV vaccination is attributed to interference of active antibody production by IBV MDA (Raggi and Lee, 1965; Davelaar and Kouwenhoven, 1977) and in this study broilers with IBV MDA were used. Based on our results, it seems that low levels of humoral antibodies did not indicate a lack of protection against IBV. In a previous study, vaccination with live H120 conferred protection against homologous challenge, although it induced low IBV antibody levels (Meir et al., 2012). Inefficient induction of antibody by live attenuated IBV vaccines has been demonstrated before (Cook et al., 1991a; Roh et al., 2013). This indicates that the resistance against IBV was due to a cellular and local tracheal immunity.

We also evaluated the effectiveness of different vaccination programmes against challenge with virulent M41, QX or 793B. Groups vaccinated with single Mass type

strains (group1 or group 2) were not challenged with virulent 793B, as it has been previously reported that Mass based vaccines did not offer protection against virulent 793B strains (Parsons et al., 1992). There was a strong degree of protection induced by the homologous and heterologous vaccination programmes against challenge with M41 or 793B. In addition, birds vaccinated with $Mass_1$+D274 (group 3) or $Mass_3$+Ark (group 6) induced full protection against the challenge with heterologous virus of 793B. It is reported that immunization with a bivalent vaccine containing Mass and Ark-type strains provided cross-protection against challenge with many field strains (Gelb et al., 1991; Martin et al., 2007), including 793B (Unpublished data cited in Jones, 2010). TOC prepared from birds vaccinated with D274 gave 93% protection against virulent 793B (Dhinakar Raj and Jones, 1996d).

The combined vaccine programme of $Mass_2$+$793B_2$ (group 5) provided high protection against the heterologous challenge with virulent IBV QX. It was previously proposed that vaccination with a live Mass-type vaccine at 1 day of age followed by a 793B vaccine two weeks later provided good protection against many heterologous IBV viruses including virulent QX (Cook et al., 1999a; Terregino et al., 2008; de Wit et al., 2011b). Despite an excellent protection against M41, QX and 793B, most early and high ciliary damages, mortality with lesions and high CD4+, CD8+ and IgA-bearing B cells were found in this group. This shows that the high degree of protection conferred against all three challenge viruses were likely due the intense cellular and local immune responses. A careful judgement between the need for a stronger protection induced by stronger immune responses against losses due to excessive clinical reactions and mortality should be considered.

Despite the low level of protection (ciliostasis test) in groups that received Mass type strains either alone or in combination with $793B_1$, D274 or Ark, the birds were fully protected against the respiratory signs caused by QX. This could be due to induction of the cellular and local immunity. To ensure effective vaccination, when developing vaccine strategies, mechanisms for inducing CTL immunity should be considered (Collisson et al., 2000).

Chapter 6: Detection of variant infectious bronchitis viruses in broiler flocks in Libya

6.1. Abstract

A number of broiler flocks with respiratory disease and high mortality in five broiler farms in Libya were sampled for detection of IBV. Twelve IBV strains from these farms were detected by RT-PCR and differentiated by nucleotide sequencing of the hypervariable region of the S1 gene. A pair-wise comparison of the sequences showed two distinctive patterns. Those from farms 1, 2, 4 and 5, formed a separate cluster with 94-99% relatedness to the Egyptian IBV strains CK/Eg/BSU-2/2011, CK/Eg/BSU-3/2011 and Eg/1212B. Sequences from the farm 3 formed another cluster with 100% relatedness to Eg/CLEVB-2/IBV/012 and IS/1494/06. This appears to be the first report on the co-circulation of variant IBVs in Libya.

6.2. Introduction

A number of IBV variant genotypes have been reported in the Middle East, including Iran/793B/19/08, Iraq /Sul/01/09, Israel/ 720/99, Israel /885/00, IS/1494/06, Egypt/ Beni-Seuf/01, Egypt/ F/03, Egypt/ D/89, CK/CH/LDL/97I, and CK/CH/SCYA/10I (Meir et al., 2004; Gelb et al., 2005; Abdel-Moneim et al., 2006; Jackwood, 2012; Ababneh et al., 2012; Kahya et al., 2013). Some of these genotypes in particular IS/885/00 and IS/1494/06 have become dominant in the majority of farms in the Middle East countries, causing respiratory and renal diseases (Meir et al., 2004; Abdel-Moneim et al., 2012; Selim et al., 2013). To date, there is no information available on the circulation of variant IBVs in Libya. In the Middle East, the vaccination against IBV is performed with vaccines that contain live attenuated belonging to the Mass serotype (Meir et al., 2004). In the past few years, vaccine strains belonging to 793B and D274 serotypes are also widely used. In spite of this, IBV infection is considered endemic and widely spread both in vaccinated and unvaccinated poultry farms generally associated with kidney damages (Meir et al., 2004; Gelb et al., 2005; Abdel-Moneim et al., 2006; Kahya et al., 2013). The aim of this study is to provide information on the molecular characteristic and the phylogenetic relationship of strains in Libya in comparison to other strains reported in the Middle East.

6.3. Materials and methods

6.3. 1. Case history and clinical samples

In July 2012, a number of broiler flocks in five different farms with respiratory disease and high mortality at East Libya were visited. The flocks had no vaccination against IBV but were vaccinated against NDV and IBD (Table 6.1). All flocks showed clinical signs of respiratory distress, manifested by sneezing, tracheal râles, gasping, nasal discharge, head swelling, conjunctival congestion and frothy eyes. Post-mortem examination revealed lesions of inflamed trachea, cheesy exudate in airsacs and swelling of the kidneys. Mortality on the day of sampling ranged from 1.4% to 3.7% (Table 6.1).

From each of the farms, OP swabs were collected from a total of 40 chicks. These swabs were divided into sets of 10 and were dipped into bijou tubes containing 2 ml of sterile water. After vigorous shaking, 100 µl of the mixture was spotted onto the FTA cards. Ten to twenty diseased birds per farm were killed and samples of turbinates, tracheas, lungs and kidneys were collected. The like-tissues were rubbed gently onto matrix areas of the FTA cards. These cards were air-dried and transported to our laboratory for RT-PCR.

6.3. 2. RNA extraction

The FTA cards were processed as described by the manufacturer. Briefly, the spotted or imprint area of the FTA card were cut using sterile scissors and forceps, each sample was placed into bijou tubes containing 2 ml of solution D (Appendix 1) and stored at -20°C until required. RNA was extracted using phenol-chloroform method as described in Chapter 2.10.i.

6.3. 3. RT-PCR and Sequencing

The RT-PCR procedure and sequences was performed as described in Chapter 2.10iii.

6.3. 3.1. Phylogenetic analysis and nucleotide comparison

Multiple sequence alignments were carried out with Clustal W, and phylogenic trees were constructed with MEGA 6 software as described in Chapter 2.11. The IBV sequences were aligned and compared with reference and vaccine strains that were used in the Middle East. The sequences were retrieved from GenBank and BLAST search was carried out. The other S1 gene sequences used for comparison or phylogenetic analysis were CK/Eg/BSU-2/2011 (JX174185), CK/Eg/BSU-3/2011 (JX174186), Eg/1212B (JQ839287), Eg/CLEVB-2/IBV/012 (JX173488), IS/885/00 (AY279533) and IS/1494/06 (EU780077), M41 (GQ219712), H120 (GU393335), D274 (X15832), 4/91 (JN600614), CR88 (JN542567) and 793B(Z83979).

6.4. Results

The daily mortality on the day sampling is given in the Table 6.1. At necropsy, the main lesions found were tracheitis, lung congestion, air-sacculitis and enlarged kidneys. IBV was detected in samples obtained from all the farms. The nucleotide sequences of these IBVs were submitted to the GenBank and the assigned accession numbers are shown in the Table 6.1. Figure 6.1and Table 6.2 shows the relatedness between the Libyan IBV sequences in comparison to those found in the Middle East and the reference IBVs. A pair-wise comparison of the IBVs, showed two distinctive patterns. The Libyan IBVs, from farm 1, 2, 4, and 5, formed a separate cluster, with 94-99% homology to CK/Eg/BSU-2/2011, CK/Eg/BSU-3/2011 and Eg/1212B.

Table 6.1. Flock details, RT-PCR and virus genotype results.

Farm	Number chicks	Age/day	Vaccination programme	Mortality/ day of sampling	RT-PCR (Laboratory sequence No, Genebank accession No)				
					OP	**Turbinate**	**Trachea**	**Lung**	**Kidney**
1	6000	32	None	2%	-	-	+,**01**,KF007922	+,**02**,KF007923	+,**03**,KF007924
2	5000	22	IBD	3%	+,**04**,KF007924	-	-	-	-
3	9000	40	NDV/IBD	3.7%	-	-	+,**05**,KF007926	-	+,**06**,KF007927
4	10000	26	IBD	2%	-	-	-	-	+,**07**,KF007928
5	7500	16	NDV	1.4%	+,**08**,KF007929	+,**09**,KF007930	+,**10**,KF007930	+,**11**,KF007932	+,**12**,KF007933

** For details, see Figure 6.1*

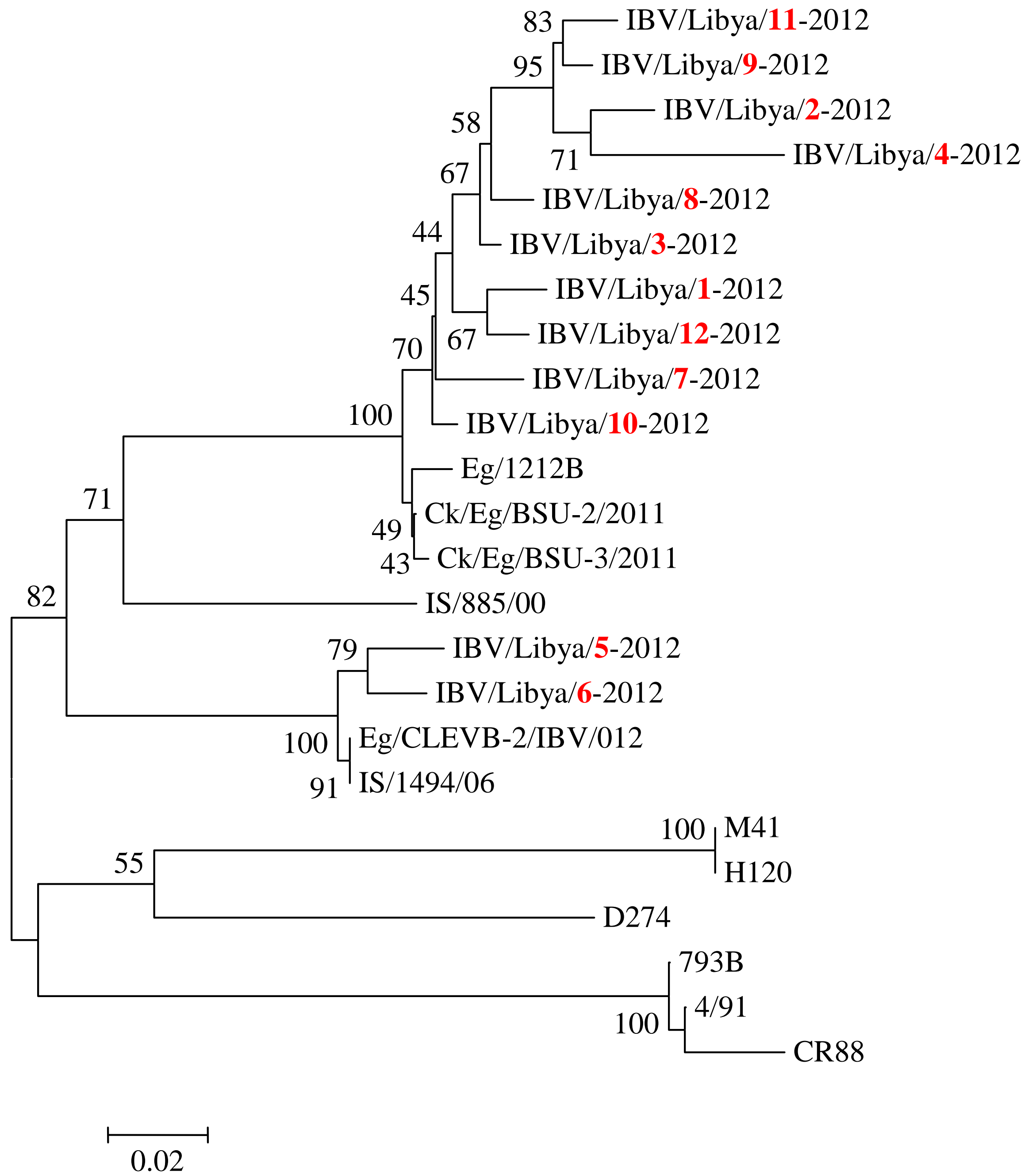

Figure 6.1. Phylogenetic tree based on a partial sequence of the S1 gene, showing the relationship between the Libyan and other IBV strains.

Table 6.2. Nucleotide and amino acid identity of the part-S1 glycoprotein gene of the Libyan in comparison to other IBV strains

% Amino acid identities																						
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20		
1		96	100	90	83	84	98	100	97	99	97	100	99	98	85	96	88	85	75	79	1	IBV/Chicken/Libya/01/12
2	99		96	94	78	79	94	96	97	95	97	96	95	94	81	91	83	81	73	74	2	IBV/Chicken/Libya/02/12
3	100	99		90	83	84	98	100	97	99	97	100	99	98	85	96	88	85	75	79	3	IBV/Chicken/Libya/03/12
4	99	98	98		74	73	88	90	91	90	91	90	89	88	74	85	77	74	68	67	4	IBV/Chicken/Libya/04/12
5	83	81	82	82		97	84	83	79	82	79	83	84	83	98	82	84	98	70	78	5	IBV/Chicken/Libya/05/12
6	83	81	82	82	100		85	84	81	83	81	84	85	84	99	83	85	99	74	79	6	IBV/Chicken/Libya/06/12
7	99	97	98	98	84	84		98	95	97	95	98	99	98	87	98	89	87	75	79	7	IBV/Chicken/Libya/07/12
8	100	99	100	98	83	83	98		97	99	97	100	99	98	85	96	88	85	75	79	8	IBV/Chicken/Libya/08/12
9	98	98	98	97	80	80	97	98		96	98	97	96	95	82	92	84	82	73	75	9	IBV/Chicken/Libya/09/12
10	100	99	100	99	83	83	99	100	98		96	99	98	97	84	95	87	84	74	78	10	IBV/Chicken/Libya/10/12
11	98	98	98	97	79	79	97	98	99	98		97	96	95	82	92	84	82	73	75	11	IBV/Chicken/Libya/11/12
12	100	99	100	99	83	83	99	100	98	100	98		99	98	85	96	88	85	75	79	12	IBV/Chicken/Libya/12/12
13	100	98	99	99	83	83	99	99	100	100	97	100		99	87	97	88	87	77	79	13	Ck/Eg/BSU-2/2011
14	99	98	99	99	83	83	99	99	99	99	97	99	100		85	97	87	85	75	78	14	Ck/Eg/BSU-3/2011
15	83	81	82	82	100	100	84	82	83	83	79	83	99	83		84	87	100	73	81	15	Eg/CLEVB-2/IBV/012
16	98	97	98	98	82	82	99	98	98	98	96	98	81	99	82		87	84	74	78	16	Eg/1212B
17	85	83	84	84	84	84	86	84	82	85	82	85	85	84	84	84		87	70	78	17	IS/885/00
18	83	81	82	82	100	100	84	82	80	83	79	83	83	83	100	82	84		73	81	18	IS/1494/06
19	70	69	70	96	73	73	71	70	69	70	68	70	73	83	73	69	72	73		68	19	Mass (H120)
20	74	72	74	71	77	77	72	73	70	74	68	74	76	77	77	73	75	77	63		20	793 B(4/91)
% Nucleotide identities																						

6.4. Discussion

The similarity to another regionally important IBV, IS/885/00, first detected in Israel, ranged from 85% to 89%. The Egyptian IBV strains CK/Eg/BSU-2 (also 3) /2011 were associated with high mortality, respiratory and renal pathology (Abdel-Moneim et al., 2012). The IS/885/00 strain was isolated in 2000 from broiler chickens in Israel. This strain was reported to cause acute renal disease, severe morbidity and high mortality ranging from 15% to 25% (Meir et al., 2004).

Those IBVs detected in farm 3 (IBV/Chicken/Libya/05/2012 and IBV/Chicken/Libya/06/2012) formed another cluster, with 100% relatedness to Eg/CLEVB-2/IBV/012 and IS/1494/06. The IS/1494/06 was first identified in Israel in 2006, was recognised as a nephropathogenic IBV, and later classified as variant 2 (R. Meir, personal communication, 2012). It has been reported that birds vaccinated with H120 were poorly protected when challenged with these strains (Meir et al., 2004; Kahya et al., 2013).

These findings show both, IS/885/00-like and IS/1494/06-like IBVs, are now circulating in Libya. Not much is known about the mode of IBV spread between the countries in the Middle East, however, cross-border movements of poultry and poultry-related products is likely an important factor. These finding shows that high mortality and severe respiratory diseases in Libyan chicken farms is likely contributed by these variant IBVs. The role of other co-infections and other exacerbating factors (eg. immunosuppression, poor ventilation, stocking density, poor management) need to be examined. In North Africa, both classical and variant IBVs have been reported in Egypt (Abdel-Moneim et al., 2006; Abdel-Moneim et al., 2012), Tunisia (Bourogaa et al., 2009) and Morocco (El-Houadfi et al., 1986). In

this study, even though only small numbers of farms were sampled, our findings have highlighted the circulation of variant IBVs in Eastern part of Libya for the first time. Further studies should include sero-surveillance, isolation and serotyping/genotyping of IBVs in the region.

Chapter 7: Immune responses and interactions following simultaneous application of live Newcastle disease, avian metapneumovirus and infectious bronchitis vaccines in specific-pathogen-free chicks

7.1. Abstract

Interactions between live NDV, aMPV and IBV vaccines following simultaneous vaccination of one-day old SPF chicks were evaluated. The chicks were divided into eight groups. One group served as unvaccinated controls, whereas the other seven groups were vaccinated against NDV, aMPV and IBV (single, dual or triple). HI NDV antibody titres were similar in single, dual or triple-vaccinated groups. Levels of aMPV ELISA antibodies were suppressed when the aMPV vaccine was given with other live vaccines. The cellular and local immunity induced by administration of live NDV, aMPV or IBV vaccines (individually or together) showed a significant increase in the expression of CD4+, CD8+ and IgA-bearing B cells in the trachea compared to the unvaccinated group. There were no significant differences between the vaccinated groups. Despite lowering the serological antibody response to aMPV, simultaneous vaccination with live NDV (VG/GA strain), aMPV (subtype B), and IBV (H120) did not affect protection against aMPV or IBV. NDV challenge was not included due to local restrictions; however, HI titres in the NDV vaccinated groups reached above the protective titre. It appears that the efficacy of each of the live vaccines was not compromised when given simultaneously (dual or triple) in young SPF chicks.

7.2. Introduction

NDV, aMPV and IBV are important and common causes of respiratory diseases in chickens. The losses due to these pathogens are significantly reduced by the use of live and inactivated vaccines worldwide, including the use of combined live vaccines. All three viruses initially replicate in the epithelium of the respiratory tract (Alexander, 2000; Jackwood and de Wit, 2013; Jones and Rautenschlein, 2013), and their ability to induce protective immunity may therefore be reduced if it is necessary to apply all three live vaccines simultaneously.

Previous *in vivo* studies, demonstrated that IBV interfered with the replication of virulent NDV (Hanson et al., 1956), live NDV vaccines (Bracewell et al., 1972; Thornton and Muskett, 1975) and aMPV vaccines in chickens (Cook et al., 2001b). Ganapathy et al. (2005a) demonstrated that NDV vaccination delayed the replication of the live aMPV vaccine, and also reduced the humoral antibody responses. These studies relied on serum antibody titres, either measured by ELISA or HI test to demonstrate the impact of simultaneous vaccine application in chicks. Systemic antibody responses have been shown to have a poor correlation with protection against IBV (Pensaert and Lambrechts, 1994) and aMPV infections (Cook et al., 1989b). Working with live NDV, IBV and aMPV vaccine viruses (Cook et al., 2001b; Ganapathy et al., 2005a; Tarpey et al., 2007), the authors suggested that the protection against these viruses was likely induced by cell-mediated and local immune responses, however this was not investigated.

It is established that cellular and local immunity plays a critical role in the protection of chicks from NDV, aMPV and IBV infections (Takada and Kida, 1996; Seo and Collisson, 1997; Rautenschlein et al., 2011). A number of studies have examined the

induction and role of CMI after vaccination with live NDV (Russell et al., 1997; Rauw et al., 2009) or IBV vaccines (Okino et al., 2013) alone. In a simultaneous vaccination study, Ganapathy et al. (2005a) reported that IgA levels in the tears of chicks that were given NDV alone or dually with aMPV were not affected. Despite the widespread application of combined live respiratory viral vaccines in young chicks, very little is known about the cellular and local immune responses following simultaneous application of live NDV, aMPV or IBV vaccine viruses. The objective of this study was to evaluate the interactions between live NDV, aMPV and IBV vaccine viruses in young SPF chicks. In addition to humoral antibody responses, the cell-mediated and local antibody responses in the trachea were also assessed. Furthermore, the protection conferred against virulent IBV and aMPV was evaluated.

7.3. Materials and methods

7.3.1. Eggs and chicks

Fertile eggs from SPF White Leghorn chickens were incubated and hatched in our facilities (Chapter 2.3.i). Chicks were placed in separate isolation units (Chapter 2.3.iii).

7.3.2. Vaccines

NDV vaccine (strain VG/GA), aMPV vaccine (subtype B), IBV vaccine (strain H120) were kindly provided by Merial SAS (Lyon France). Vaccines were reconstituted as recommended by the manufacturer and the titres are estimated from data provided by the manufacturer. For NDV and aMPV vaccines, each 1,000 dose vial was reconstituted in 2 ml of chilled SW and thoroughly mixed with 100 ml of SW. For IBV vaccine, a 5,000 dose vial was reconstituted in 5 ml of SW and then 1 ml was mixed with 100 ml of SW. For dual-vaccination, NDV vaccine was first reconstituted and then followed by aMPV or IBV vials as previously described. Each pair was then mixed in 100 ml SW. For triple vaccination, NDV, aMPV and IBV were reconstituted as above and all three were diluted in 100 ml of SW.

7.3.3. Challenge viruses

7.3.3. 1. IBV

IBV M41 strain was grown in ECE and titrated in TOC as described (Chapter 2.8). The titre obtained was $10^{5.00}$ CD_{50}/ml.

7.3.3. 2. aMPV

aMPV subtype B virus was propagated and titrated in TOC (Chapter 2.8). The titre obtained was $10^{4.51}$ CD_{50}/ml.

7.4. Experimental design

Three hundred and twenty 1-day old chicks were randomly distributed in 8 groups, each group consisting of 40 chicks per isolation unit. The vaccination treatments consisted of a single dose of NDV, aMPV or IBV vaccines, or dual or triple combinations of these vaccines (Table 7.1). Each chick was vaccinated via oculo (50 µl) nasal (50 µl) route. Dosages received by each bird were as recommended by the manufacturers (Table 7.1). Following the vaccination, the chicks were observed daily for the clinical signs. OP swabs were randomly collected prior to vaccination and from 10 chicks in each group at 3, 7, 14, 21, 26 and 35 dpv for RT-PCR. For serology, blood was collected prior to vaccination and at 21 and 35 dpv from eight chicks in each group. At 21 dpv, five chicks from each group were humanely killed and trachea samples were collected from each bird, immediately placed in aluminium foil cups containing OCT medium and frozen in liquid nitrogen (-190°C). These tracheas were used for detection of CD4+, CD8+ and IgA-bearing B cells by IHC. All samples were stored at -70 °C until processing. At 21 dpv, 10 birds from each group were transferred to a different isolation room and one was challenged with 0.1 ml virulent aMPV subtype B by the oculonasal routes. An additional 10

birds from each group were challenged with 0.1 ml virulent IBV M41 via the same route. The remaining 15 chickens in each group were left as unchallenged.

Table 7.1. Experimental design for the single and simultaneous application of dual or triple live NDV, aMPV and IBV vaccines in SPF chicks.

Groups	Vaccines	Oculo-nasal dose
1	NDV	5.5 log_{10} EID_{50}/chick
2	aMPV	2.3 log_{10} $TCID_{50}$/chick
3	IBV	3.5-5.0 log_{10} EID_{50}/chick
4	NDV+aMPV	5.5 log_{10} EID_{50}/chick+2.3 log_{10} $TCID_{50}$/chick
5	NDV+IBV	5.5 log_{10} EID_{50}/chick+3.5-5.0 log_{10} EID_{50}/chick
6	aMPV+IBV	2.3 log_{10} $TCID_{50}$/chick3.5-5.0 log_{10} EID_{50}/chick
7	NDV+aMPV+ IBV	5.5 log_{10} EID_{50}/chick+2.3 log_{10} $TCID_{50}$/chick+3.5-5.0 log_{10} EID_{50}/chick
8	Unvaccinated control	SW

7.4.1. Detection of vaccine viruses by RT-PCR

Pooled daily swabs were dipped as a single sample per group, per day in a labelled bijou that contained 1.5 ml of solution D (Appendix 1). RNA was extracted using the phenol-chloroform method (Chapter 2.10.i). RT-PCR was performed for NDV, aMPV and IBV as described in Chapter 2.10.iii.

7.4.2. Serology

NDV antibodies were detected by HI (This was done in commercial laboratory, SciTech laboratories, The Grove, Craven Arms, Shropshire SY7 8DA). For aMPV and IBV, sera were tested by commercial ELISA (BioChek) (Chapter 2.12.i.a).

7.4.3. Immunohistochemical analysis of cell-mediated responses in tracheal sections

Indirect immunohistochemical staining was conducted to quantify immune cell populations of CD4+, CD8+ or IgA-bearing B cells (Chapter 2.13).

7.4.4. Assessment of protection against aMPV challenge

Following aMPV challenge, chicks were observed daily up to 13 dpc to record the clinical signs. Ten chicks from each group were examined individually as described in Chapter 2.5.ii.

7.4.5. Assessment of protection against IBV challenge

Following IBV challenge, all chicks were observed daily for clinical signs attributable to IBV infection. At 5dpc, the chicks were humanely killed for gross lesions (Chapter 2.5.iii) and determine level of protection of the trachea using the ciliostasis test (Chapter 2.5.v).

7.4.6. Statistical analysis

The data of the serological responses, CMI and aMPV clinical signs were analysed by one way ANOVA followed by Tukey's test. Differences were considered to be significant when $P \leq 0.05$. All analysis were conducted using the GraphPad Prism, 6.0.1.

7.5. Results

7.5.1. Clinical signs post-vaccination

No clinical signs were observed in any of the vaccinated groups.

7.5.2. Detection of vaccine viruses by RT-PCR

None of the viruses was detected in pooled swabs from any group prior to the vaccination.

NDV was detected at 3 dpv only in the single and dual-vaccinated NDV groups, and up to 7 dpv in the triple vaccinated group (Table 7.2). aMPV was detected up to 7 dpv in birds that received single vaccine and 14 dpv in those given NDV and aMPV vaccines. In contrast, aMPV was detected for a much longer duration (up to 21 dpv) when administered with IBV or with NDV and IBV vaccines. In all IBV-vaccinated groups, IBV was detected to the end of the experiment.

7.5.3. Detection of viruses after challenge

Virulent aMPV subtype B was detected at 6 but not at 13 dpc in groups that were not vaccinated against aMPV (Table 7.2). aMPV was not detected in groups that received aMPV vaccine, whether singly or in dual or triple combinations.

Table 7.2. RT-PCR detection of vaccine viruses following vaccination of chickens with NDV, aMPV and IBV vaccines.

Vaccines			**dpv**						**dpc**	
NDV	**aMPV**	**IBV**	**3**	**7**	**14**	**21**	**26**	**35**	**6≠**	**13≠**
+			N*	-	-	-	-	-	+	-
	+		M	M	-	-	-	-	-	-
		+	B	B	B	B	B	B	+	-
+	+		NM	-M	-M	--	--	--	-	-
+		+	NB	-B	-B	-B	-B	-B	+	-
	+	+	MB	-B	-B	MB	-B	-B	-	-
+	+	+	NMB	NMB	--B	-MB	--B	--B	-	-
Unvaccinated Control			---	---	---	---	---	---	+	-

N: Positive for NDV vaccine; M: Positive for aMPV vaccine; B: Positive for IBV vaccine; * *Ten OP swabs pooled as single sample;* ≠ *OP swabs collected following aMPV challenge.*

7.5.4. Detection of NDV HI antibodies

HI titres in groups not vaccinated with NDV vaccine remained below the detectable levels. At 21 and 35 dpv, chicks vaccinated with NDV vaccine either alone or in combination had significantly higher antibody titres than chicks that did not vaccinated with NDV vaccine (Figure 7.1). There were no significant differences in the level of HI antibody titres between the groups that received NDV vaccine.

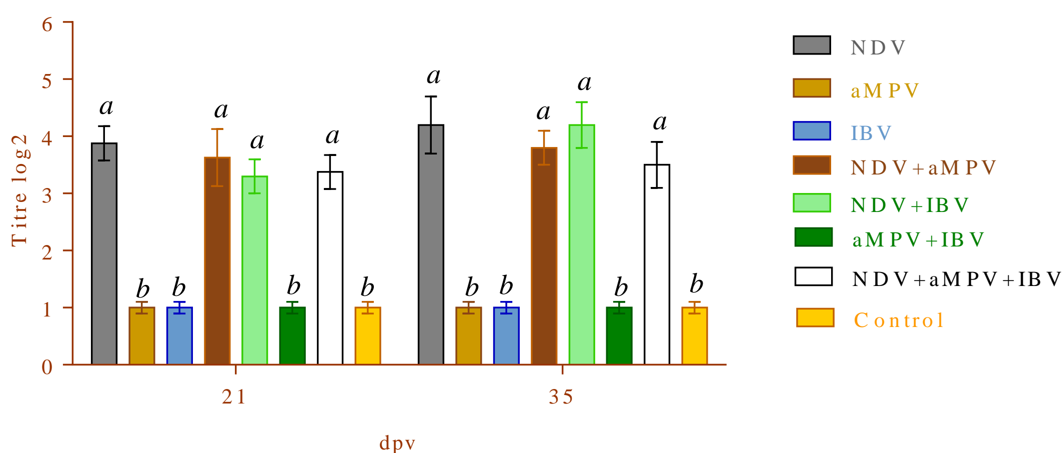

Figure 7.1. NDV HI antibody titres in chicks administered with live NDV vaccine alone or with other live vaccines. Vertical bars represent SEM (n= 8). Different superscripts letters represent significant differences in the antibody response, while bars with the same superscript letters are not significantly different.

7.5.5. Detection of aMPV antibodies by ELISA

Sera collected prior to vaccination or from the aMPV unvaccinated groups did not have detectable of level aMPV antibodies. At 21dpv, chicks vaccinated with aMPV vaccine singly showed significantly higher levels of antibody titre than chicks vaccinated with aMPV vaccine either with NDV and/or IBV (Figure 7.2). At 35 dpv, antibody titres in the single aMPV vaccinated chickens remained higher than in the dual or triple vaccinated chicks (Figure 7.2).

At 21 and 35 dpv chicks vaccinated with aMPV vaccine either alone or in combination had significantly higher antibody titres than chicks that did not vaccinate with aMPV vaccine.

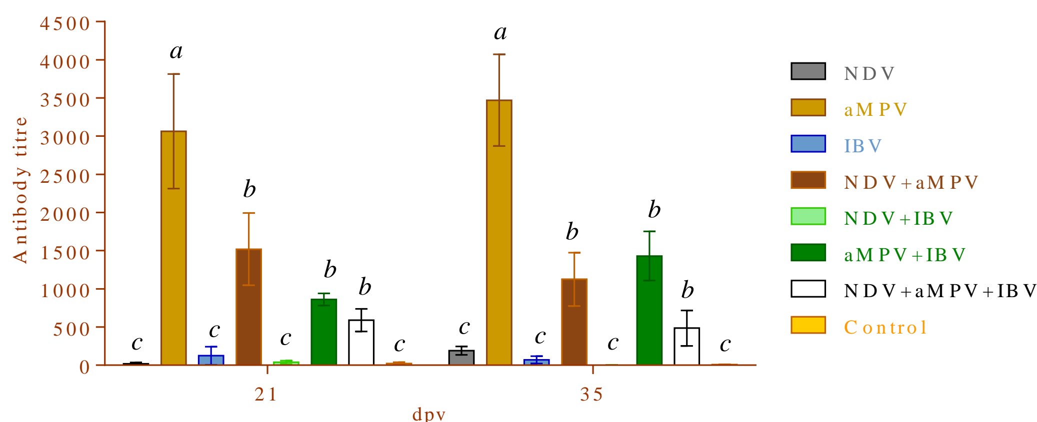

Figure 7.2. aMPV ELISA antibodies in chicks vaccinated with aMPV or with NDV and/or IBV. Vertical bars represent SEM (n= 8). Different superscripts represent significant differences in the antibody response, while bars with the same superscript letters are not significantly different. The cut-off titre=1656.

7.5.6. Detection of IBV antibodies by ELISA

There were no IBV antibodies detected in sera collected prior to vaccination or from those groups not vaccinated with IBV vaccine. Chicks vaccinated with H120 vaccine alone or in combination with other vaccines showed high levels of antibody titre against IBV compared to unvaccinated groups (Figure 7.3). However, there were no significant differences in the level of antibody titres between the groups that received H120 vaccine.

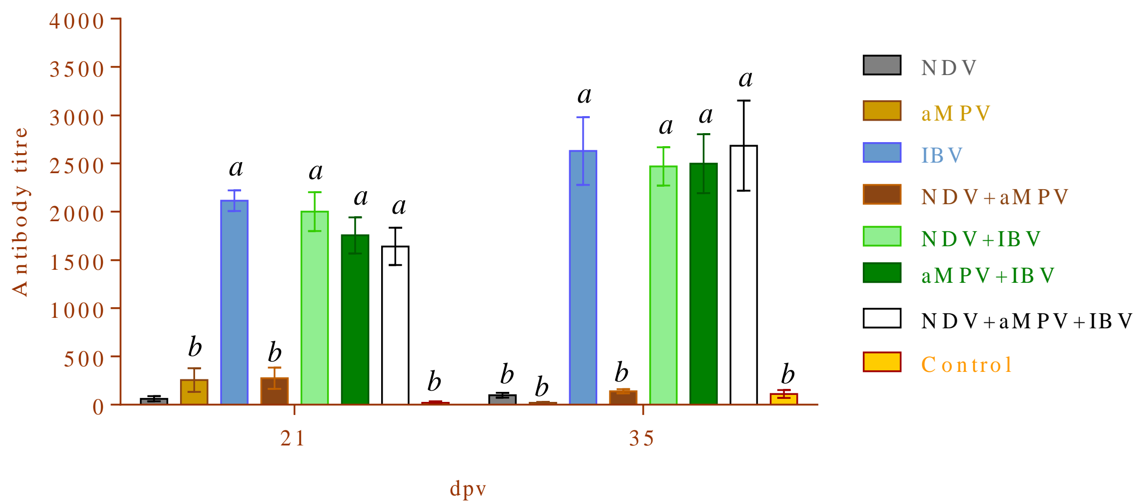

Figure 7.3. IBV ELISA antibodies in chicks vaccinated with IBV alone or with NDV and/or aMPV. Vertical bars represent SEM (n= 8). Different superscripts represent significant differences in the antibody response, while bars with the same superscript letters are not significantly different. The cut-off titre= 834.

7.5.7. CD4+, CD8+ and IgA-bearing B cells in the trachea

The average numbers of CD4+, CD8+ and IgA bearing B-cells in the trachea were evaluated by IHC. Single, dual or triple vaccinated groups showed significantly greater expression of CD4+, CD8+ (Figure 7.5) and IgA bearing B-cells (Figure 7. 6) in tracheal tissue compared to the unvaccinated control groups. However, there were no significant differences between the vaccinated groups at 21dpv. Figure 7.8 shows the immunostaining of trachea sections of unvaccinated control birds whereas Figure 7.9 shows immunostaining of trachea sections of vaccinated birds.

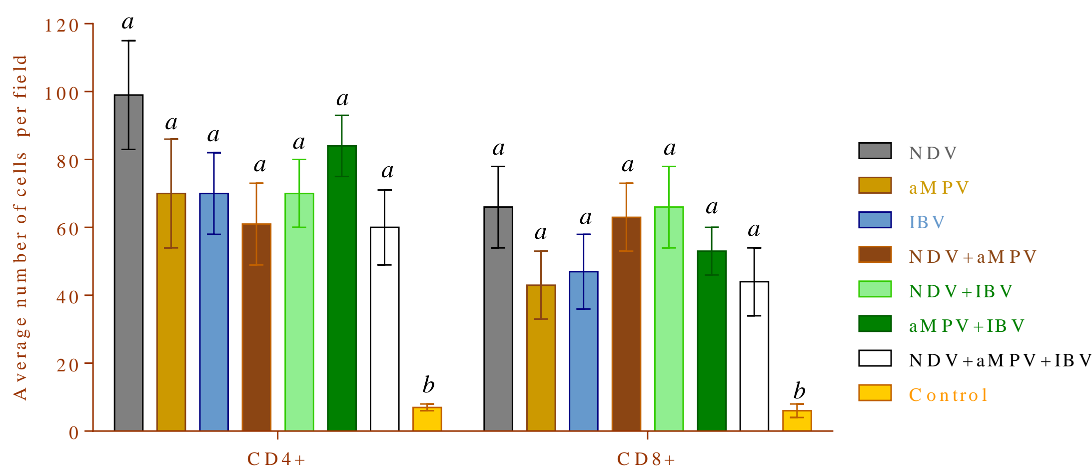

Figure 7.5. Immunostaining of trachea sections following vaccination using Mabs for presence of CD4+ and CD8+ T-cells. Vertical bars represent SEM (n= 5). Different superscripts represent significant differences in the antibody response, while bars with the same superscript letters are not significantly different.

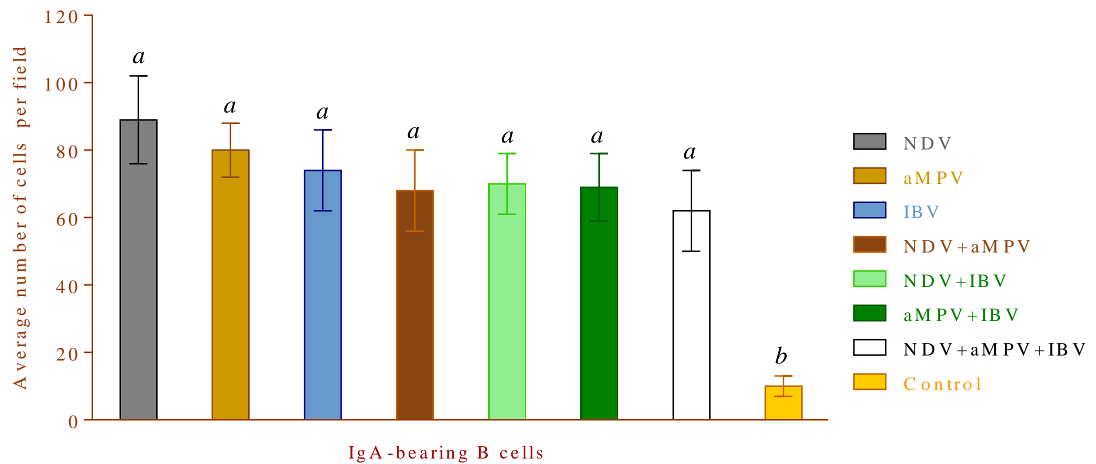

Figure 7.6. Immunostaining of trachea sections following vaccination using Mabs for presence of IgA-bearing B cells. Vertical bars represent SEM (n= 5). Different superscripts represent significant differences in the antibody response, while bars with the same superscript letters are not significantly different.

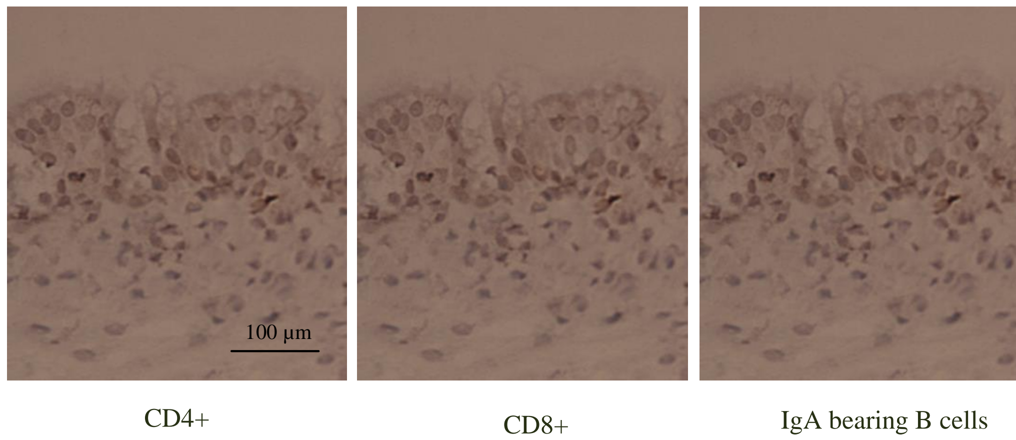

Figure 7.8. Immunostaining of trachea sections of unvaccinated control birds

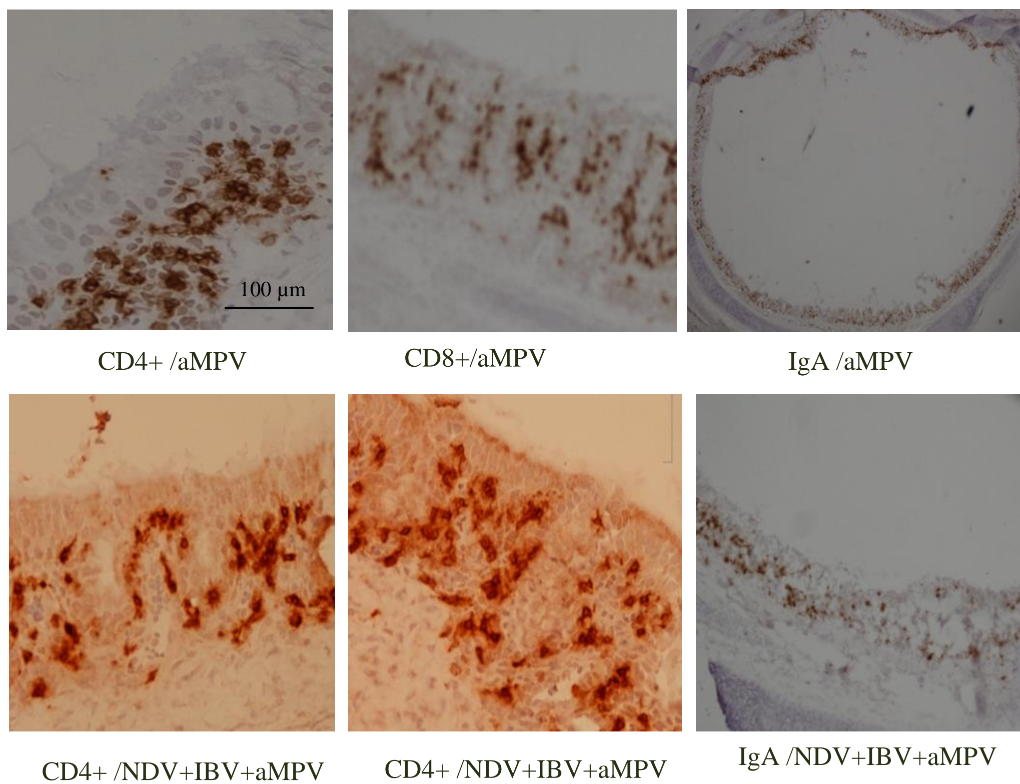

Figure 7.9. Immunostaining of trachea sections following single or triple vaccination

7.5.8. Effects of NDV and IBV vaccinations on protection against aMPV challenge

7.5.8. 1. Clinical signs

Following virulent aMPV challenge, no clinical signs were observed in the groups vaccinated with aMPV alone or co-delivered with NDV or IBV, or both (Figure 7.10). In contrast, chicks not vaccinated against aMPV showed clinical signs such as clear to turbid nasal discharges at 3 dpc and completely recovered by 11 dpc. No significant differences in clinical signs were seen in the groups that did not receive aMPV vaccine.

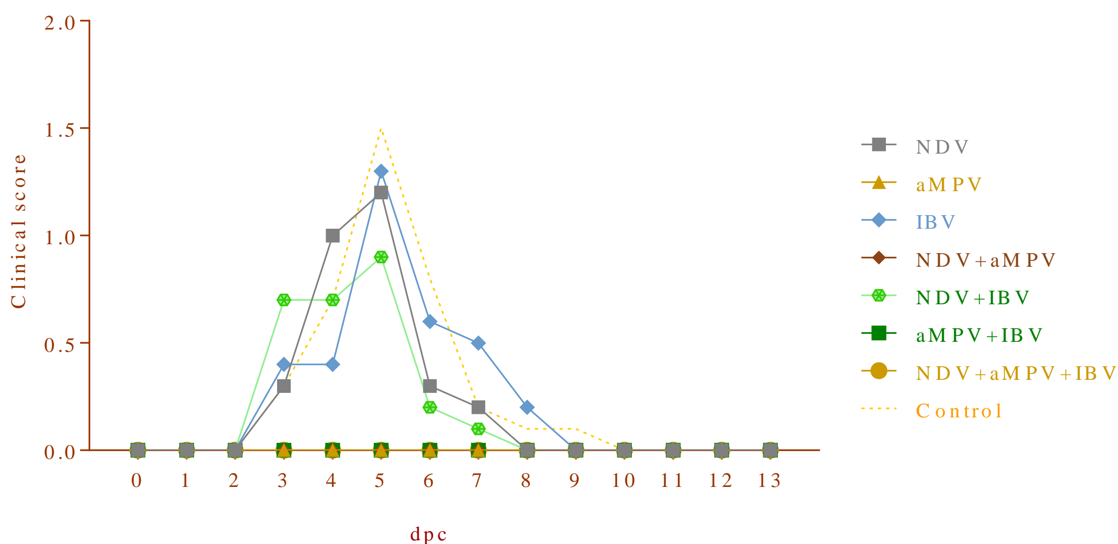

Figure 7.10. Mean clinical scores in the vaccinated and unvaccinated chickens after aMPV challenge. No clinical signs were observed in the chickens vaccinated with aMPV vaccine alone or together with NDV or IBV or both.

7.5.9. Effect of NDV and aMPV vaccination on protection against IBV challenge

7.5.9.1. Clinical signs and gross lesions

Following IBV challenge, no clinical signs were observed in the group vaccinated with H120 vaccine alone or in combination with the other vaccines. Chicks not vaccinated with H120 vaccine showed mild respiratory signs at 1dpc which included tracheal râles, coughing, sneezing and head shaking. At necropsy at 5 dpc, chicks vaccinated with H120 vaccine were found to be free of lesions, while unvaccinated chicks had slight congestion in the trachea and pale and mild swelling of kidneys.

7.5.9.2. TOC assessment for protection against IBV challenge

Virulent IBV M41 caused substantial damage to the tracheal epithelium of the chicks not vaccinated with H120 vaccine. Groups that were given H120 vaccine showed 95-98% protection against IBV M41 (Figure 7.11). In the vaccinated/unvaccinated unchallenged groups, almost 100% the ciliary protection were recorded.

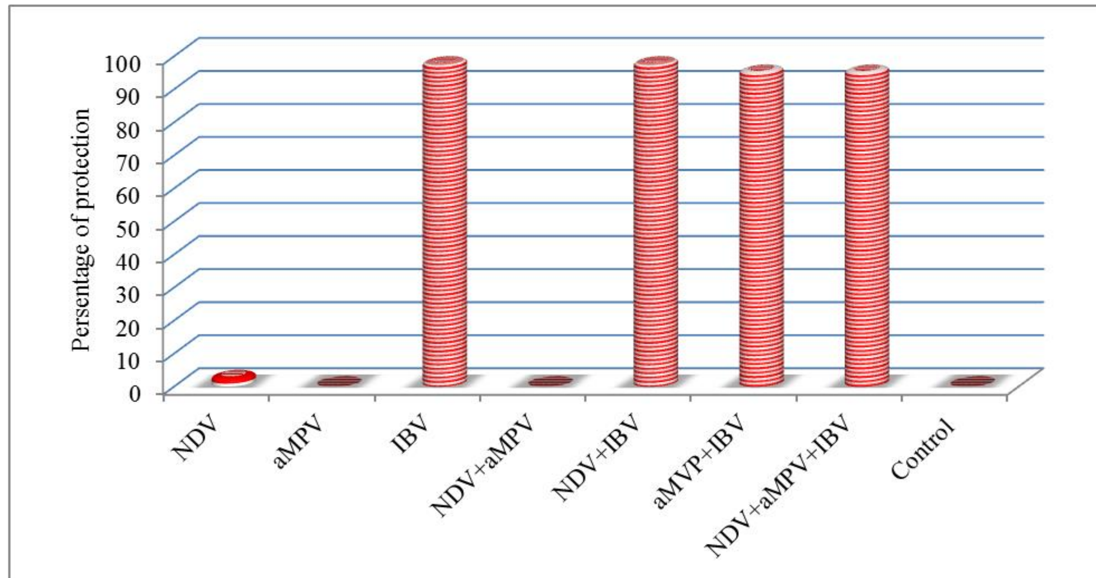

Figure 7.11. Protection against IBV M41challenge of chicks vaccinated with IBV H120 vaccine alone or together with NDV and/ or aMPV.

7.6. Discussion

We evaluated the effect of co-delivering live NDV, aMPV and IBV vaccine viruses in combination in SPF chicks. Previous studies have reported the simultaneous application of NDV and aMPV (Ganapathy et al., 2005a; Ganapathy et al., 2006), aMPV and IBV (Cook et al., 2001b), or NDV, aMPV and IBV (Tarpey et al., 2007) in chicks. Those studies reported that simultaneous application of live NDV or IBV vaccines reduces humoral antibody responses to virulent aMPV, but the chicks were protected against disease. The authors strongly suggested that CMI may have played an important role in conferring the protection against challenge viruses of NDV, aMPV or IBV but this was not investigated. In the current comprehensive study, in order to generate comparative data, the same strains of live NDV, aMPV and IBV vaccine viruses were given to young SPF chicks in single, dual or triple combinations. In addition to humoral antibody responses, we monitored the cell-mediated and local immune responses in the trachea of the birds using IHC.

Following single and simultaneous (dual and triple) application of the live vaccine viruses, to one day old SPF chicks no respiratory or any other clinical signs were found. Similar observations were reported when live NDV and aMPV (Ganapathy et al., 2007), aMPV and IBV (Cook et al., 2001b) and NDV+IBV and aMPV (Tarpey et al., 2007) were concurrently administered to SPF chicks. In the previous studies (Cavanagh et al., 1999; Cook et al., 2001b; Ganapathy et al., 2005a; Ganapathy et al., 2006) the distribution and persistence of the live vaccine viruses in various tissues and OP swabs were monitored. In our study, we used RT-PCR to trace the vaccine viruses in OP swabs and it was noted that the live IBV vaccine virus was detected throughout the experimental duration. This demonstrates the persistence of

IBV, which also could be related to its ability to dominate the other live respiratory vaccines. Cook et al. (2001b) reported that this is likely due the rapid replication of IBV. In our experience, under experimental and field conditions, we are able to detect IBV vaccine viruses by RT-PCR for many weeks following vaccination (Cavanagh et al., 1999; De Wit et al., 2010a).

For the NDV vaccine, it appears that the virus is cleared rapidly when given alone or simultaneously with IBV or aMPV, but lasted slightly longer (up to 7 dpv) when all three vaccine viruses were given together. The aMPV vaccine virus could be detected in OP swabs for a much longer (up to 21 dpv) duration when administered with IBV or in combination with IBV and NDV. Prolonged detection of aMPV after dual vaccination with NDV has been reported before (Ganapathy et al., 2005a). There appears to be a delayed detection of NDV or aMPV when co-delivered with live IBV vaccine virus, potentially indicating the dominance of IBV virus. Despite this, it is evident that the NDV or IBV vaccine viruses are not completely eliminated, and instead allowed to replicate at a later time when co-administered with IBV.

An important indication of successful vaccine-take is a serological response, as this indicates the ability of the vaccine to attach, replicate and induce immune responses, including humoral antibodies. In this study, IBV and aMPV humoral antibodies were measured using commercial ELISA kits and HI was used for detection of NDV antibodies. For IBV and NDV, the results demonstrate that simultaneous dual or triple vaccinations did not affect the levels of the antibodies induced. This is particularly important for NDV, as HI antibody levels are commonly used as an indicator of protection (Goddard et al., 1988; Reynolds and Maraqa, 2000; Carrasco et al., 2009; Ezema et al., 2009). For aMPV, the lower humoral antibody titres in

groups that were given dual or triple vaccinations were at the same level as previously reported (Cook et al., 2001b; Ganapathy et al., 2005a; Tarpey et al., 2007).

There are currently no published data on the cell-mediated responses to dual or triple vaccinations, even though such programmes are increasingly practiced in the poultry industry worldwide. We attempted to measure the CD4+ and CD8+ responses in the trachea following single, dual or triple vaccinations. In addition, the trachea was also assessed for IgA-bearing B-cells as an indicator of the local immune response. Responses were evaluated at 21 dpv before the flocks were separated for challenge against IBV or aMPV. Our findings showed significant increases in the expression of CD4+, CD8+ or IgA-bearing B-cell in the trachea from vaccinated compared to unvaccinated groups. The absence of significant differences between the vaccinated groups demonstrates that at the tracheal level, similar intensities of cell-mediated and local immune responses were induced by the single, dual and triple vaccinations. In contrast, as previously discussed, there was suppression in the serological response to aMPV but not for IBV or NDV.

As part of the study, the efficacy of live vaccines when given simultaneously (dual or triple) against single application were compared. For IBV and aMPV, the protections conferred against challenge viruses were evaluated. For NDV, due to local regulations, no challenge was carried out; instead NDV HI titres following vaccination were used. Our results showed that irrespective of live vaccine combinations, protection against IBV was not affected, as ciliary protection of 95-98% was achieved in all IBV-vaccinated groups. Similar findings have previously been shown in other *in vivo* studies (Cook et al., 2001b; Gelb et al.,

2007). For NDV, mean HI titres of 2-5 log_2 and above were considered to provide clinical protection (Reynolds and Maraqa, 2000; Ganapathy et al., 2007; van Boven et al., 2008; Shim et al., 2011; Jeong et al., 2013). In this study, it appears that simultaneous application of live NDV vaccine with aMPV, IBV or both does not compromise the immune response or protection conferred against NDV.

With increasing losses due to aMPV infection in broiler, layer and breeder chickens, live vaccines against aMPV are applied, often along with live IBV, NDV or both. Previous work has shown that protection against aMPV was not compromised when the live aMPV vaccine was given with live IBV (Cook et al., 2001b), NDV (Ganapathy et al., 2005a) and NDV+IBV combined vaccine (Tarpey et al., 2007). Results from this study demonstrate no loss of protection when the aMPV vaccine was given either alone, or in combination. Such protection was achieved irrespective of the humoral antibody levels. This finding supports previous reports contending that humoral antibodies do not have a major role in protection against aMPV challenge (Jones et al., 1992; Naylor et al., 1997b). In a long-term experimental study, turkey hens given a live aMPV vaccine at 12 days of age were protected against challenge at 22 weeks, even with no significant levels of ELISA antibody at the time of challenge (Williams et al., 1991). This demonstrates that antibody detection is an insufficient parameter for estimating the degree of protection in aMPV vaccinated poultry flocks (Rubbenstroth and Rautenschlein, 2009). Our study confirms previous suggestions that the protection against aMPV was due to cell mediated and local immune responses, as the levels of CD4+, CD8+ and IgA-bearing B-cells were similar in all aMPV-vaccinated groups.

Based on the findings presented in this study, it appears that the efficacy of the live NDV, aMPV and IBV vaccine viruses were not compromised when given simultaneously in dual or triple combinations to young SPF chicks. There was a suppression of humoral antibody responses to aMPV, but responses to IBV and NDV were not affected. At the tracheal level, no variations in the levels of cell mediated or IgA-bearing B-cells were seen between vaccinated groups.

Chapter 8: Comparative pathogenesis of avian metapneumovirus subtypes in specific pathogen-free chicks

8.1. Abstract

Most aMPV infection studies have been done with a single subtype, using either A or B in turkeys or chickens. Little is known about co-infection of these two subtypes in SPF chicks. In this study, virulent subtypes A and B were used to infect SPF chicks, either singly or in combination (A+B). Clinical signs, virus distribution in tissues and sero-conversion were examined. There were significant differences in the degree of the clinical signs induced by the single subtypes A, B or A+B given together, with most severe signs observed in the latter two groups. By RT-PCR or VI, subtype B virus persisted longer than the subtype A. Tissue samples were positive for subtype A and B for up to 5 and 14 dpi respectively by RT-PCR and 3 and 7 days respectively by VI. OP and CL swabs were positive for subtype A for up to 5 dpi but up to 9 dpi for B. Even though similar titres of the viruses were used, birds given subtype B alone or in combination showed a greater increase in ELISA antibody titres than those given A. These findings demonstrate that for the two strains used, subtype B was more pathogenic than A and was excreted and persisted in the tissues for longer.

8.2. Introduction

Avian metapneumovirus, also known as turkey rhinotracheitis virus, causes a widespread disease of turkeys, chickens and few other avian species (Cook and Cavanagh, 2002), characterized by an upper respiratory tract infection. aMPV is also associated with SHS in broilers and broilers breeders (Cook et al., 1988; Jones et al., 1991; Gough et al., 1994) and egg production losses in layers turkeys and chickens (Hafez et al., 1990; Cook et al., 1996b; Sugiyama et al., 2006). aMPV was first described in turkey in South Africa in 1978 (Buys et al., 1989), and since then the virus has been reported in Europe, United States, Middle East, Asia and Africa and is now considered to be a major disease threat in both turkeys and chickens in many parts of the world (Collins et al., 1993; Seal, 1998; Cook et al., 1999b; Bäyon-Auboyer et al., 2000; Hafez et al., 2000; Mase et al., 2003; Banet-Noach et al., 2005; Chacón et al., 2011).

Several pathogenesis studies have been reported with aMPV subtype A or B in turkeys or chickens (Jones et al., 1988; Cook et al., 1993; Majó et al., 1995; Liman and Rautenschlein, 2007; Aung et al., 2008). To the best of our knowledge, there are no comparative studies in SPF chicks using both aMPV subtype A or B, separately and in combination. The objective of the present study was to compare the development of the disease caused by aMPV A, and B or mixture of A and B in SPF chicks. Clinical signs, virus detection and antibody responses were examined.

8.3. Materials and methods

8.3.1. Eggs and chicks

Fertile eggs from SPF White Leghorn chicken were incubated and hatched at our facilities (Chapter 2.3.i).

8.3.2. Viruses

aMPV subtypes A and B. Both were propagated and titrated in TOC (Chapter 2.8.ii). The median ciliostasis doses for subtypes A and B were $10^{4.56}$ CD_{50}/ml and $10^{4.51}$ CD_{50}/ml respectively.

8.4. Experimental design

One hundred and sixty, seven-day old SPF chicks were randomly divided into four groups with 40 birds in each group. Group A birds were inoculated oculonasally with 0.1ml of subtype A virus. Group B birds were inoculated with 0.1ml of subtype B in the same manner. Group A+B birds were inoculated with 0.1 ml of subtype A via the left nostril and left eye, and 0.1 ml of subtype B via the right nostril and right eye. Group C (the control group) birds were inoculated with 0.1 ml virus-free TOC medium. At 1, 3, 5, 7, 9 and 14 dpi, five chicks from each group were swabbed (OP and CL) and then were humanely killed to allow collection of tissue samples (turbinate, trachea, lung and spleen) for virus detection by RT-PCR and VI. Serum samples were collected from ten birds per group before inoculation and at 14 dpi to detect antibodies against aMPV infection by two different commercial ELISA kits.

8.4.1. Assessment of clinical signs

Following aMPV infection, birds were observed once daily up to 14 dpi to record clinical signs. Ten birds from each group were examined individually described in Chapter 2.5.ii.

8.4.2. Sample collection for RT-PCR and VI

Samples of turbinate, trachea, lung and spleen were collected aseptically and then were pooled per organ, per group and ground up using a pestle and mortar (Chapter 2.9.iii).

8.4.3. OP and CL swabs for RT-PCR and VI

Like swabs were dipped together as a single sample in 1.5 ml of TOC medium (Chapter 2.9.ii) and then supernatants were stored at -70°C until required.

8.4.4. Detection of aMPV by RT-PCR

RNA was extracted using phenol-chloroform method as described in Chapter 2.10i. The aMPV genome was detected by RT-PCR based on the attachment protein gene (G) (Chapter 2.10.iii). Using subtype specific PCRs whereby common primer G5- is paired with G8+A for subtype A specificity and G9+B for subtype B specificity. Subtype A and B viruses result in bands of 268 bp and 361 bp respectively and are readily distinguished on agarose gels.

8.4.5. VI

Virus isolation was performed in chicken embryo TOC (Chapter 2.8.ii); using three replicate cultures per sample and up to three 10-day passages. Ciliostasis was taken as indicator of aMPV detection (Chapter 2.5.v). aMPV identification and subtyping were determined by RT- PCR (Chapter 2.10.iii).

8.4.6. Detection of aMPV ELISA antibodies

Two commercial ELISA kits (referred here as Kit 1 and Kit 2) were used to detect antibodies against aMPV as recommended by the respective manufacturers of the kits.

8.4.7. Statistical analysis

The mean antibody titres of the infected and clinical scores were analysed by one way ANOVA followed by Tukey's test, using GraphPad Prism, 6.01.

8.5. Results

8.5. 1. Clinical signs

During the experiment, the control group inoculated with TOC medium only remained free of clinical signs (Figure 8.1). The clinical sign recorded was the presence of clear to turbid nasal exudates in some birds. Groups B and A+B both showed signs as soon as 2 dpi with similar peaks around 4 and 5 dpi. However, for group A+B recovery was by 12 dpi, which was two days longer than group B. Clinical signs in groups A did not appear until day 3 and they subsided sooner than A+B (Figure 8.1). The birds inoculated with subtype B either alone or with subtype A, produced significantly higher clinical sign scores than birds inoculated only with subtype A on days 1, 2, 3, 4, 6 and 7 post-infection but none thereafter. However, there was no significant difference between groups given subtype B alone or together with A except on day 6 (Figure 8.1). There was no significant difference on day 8, 9 (aMPV A), day 9 (aMPV B) and day 11 (aMPV A+B) with control group. Chicks in all groups were fully recovered by 12 dpi. No mortalities were recorded in any of the infected groups throughout the experiment.

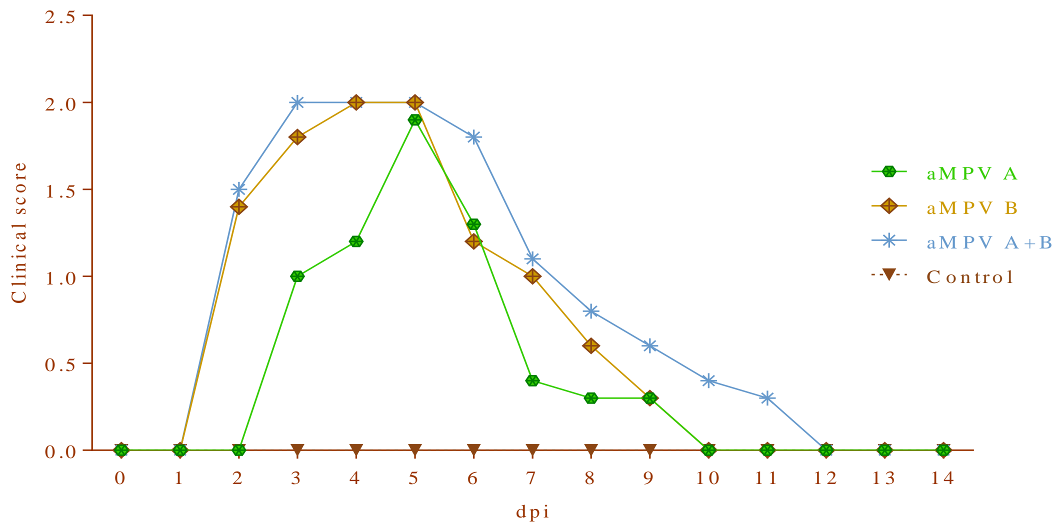

Figure 8.1. Mean clinical sign scores following aMPV subtype A, subtype B or combination of A+B infection in SPF chicks.

8.5. 2. Detection of aMPV by RT-PCR and VI

8.5. 2. 1. Swabs

No viruses were detected in the control birds throughout the experimental period. Table 8.1 shows the results of the detection of viral shedding using OP and CL swabs. In group A, viral genome was detected up to 5 and 7 dpi in OP and CL swabs respectively but virus was isolated only on day 3 in CL swabs. In group B, viral RNA was detected in both sets of swabs though intermittently from the CL, for up to 9 dpi but isolated in only from that site on day 5. In group A+B only virus B was detected and then only in OP swabs on 3 dpi by PCR only.

Table 8.1. Detection of aMPV in swabs by RT-PCR and VI in TOC.

Swabs	Groups	dpi					
		1	**3**	**5**	**7**	**9**	**14**
OP	A	+	+[VI]	+	-	-	-
	B	+	+	+	+	+	
	A+B	- /-[*]	-/+	-/-	-/-	-/-	-/-
CL	A	-	-	+	+	-	-
	B	-	-	+[VI]	-	+	-
	A+B	- /-	-/-	-/-	-/-	-/-	-/-

Control chicks free of aMPV throughout the experimental period; + aMPV positive by RT-PCR; - aMPV negative by RT-PCR; [VI] Positive for VI; [*]= aMPV A/ aMPV B.

8.5. 2. 2. Tissues

The distribution of aMPV in different tissue samples is shown in Table 8.2. For group A, virus was detected by RT-PCR up to day 5 consistently from turbinate and trachea but only less so from lung and spleen. None was isolated at any time. In group B, the virus was consistently detected in the trachea for up to 5 dpi. However, in the turbinate, lung and spleen, subtype B was still being detected up to 14 dpi. The virus was only isolated from the lungs at 1 dpi and the turbinate at 3 dpi. In group A+B, only subtype B was detected in the tissues, but not in spleen. The turbinate was positive up to 9 dpi whereas the trachea and lung were positive until 5 and 3 dpi respectively. VI of B was successful in six samples taken from the birds infected with both subtypes with virus isolated as long as 7 dpi in the turbinate of these birds.

Table 8.2. Detection of aMPV in tissue by RT-PCR and VI in TOC.

Tissue samples	Groups	dpi					
		1	**3**	**5**	**7**	**9**	**14**
Turbinate	A	+	+	+	-	-	-
	B	-	+[VI]	-	+	+	+
	A+B	- /+[*]	-/+	-/-	-/+[VI]	-/+	-/-
Trachea	A	+	+	+	-	-	-
	B	+	+	+	-	-	-
	A+B	-/+[VI]	-/+[VI]	-/+[VI]	-/-	-/-	-/-
Lung	A	-	+	+	-	-	-
	B	+[VI]	+	+	-	+	+
	A+B	-/+	-/+[VI]	-/-	-/-	-/-	-/-
Spleen	A	-	-	+	-	-	-
	B	-	+	+	-	+	+
	A+B	-/-	-/-	-/-	-/-	-/-	-/-

Control chicks free of aMPV throughout the experimental period. + aMPV positive by RT-PCR; - aMPV negative by RT-PCR; [VI] Positive for VI; [*] aMPV A/ aMPV B.

8.5.3. aMPV ELISA antibodies detection

Mean ELISA titres at 14dpi using two different ELISA kits from two different manufacturers is shown in Figure 8.2 (Kit 1) and Figure 8.3 (Kit 2). In both ELISA kits, serum samples collected prior to infections and control birds were free of the aMPV antibodies. In both ELISA kits, all infected birds, either singly (A or B) or in combination (A+B) showed high significant antibody titres against aMPV compared to control birds. Groups of birds that infected with subtype B either singly or as part of a co-infection with subtype A showed high antibody titres compared to the birds infected with subtype A alone. The highest titres were seen in both subtype infected birds and there were significant differences ($P > 0.05$) between subtype B-infected birds and the group of birds infected with both subtypes A+B. In ELISA kit 1, based on the recommended cut-off value, subtype A infected birds had a low mean antibody titres (Figure 8.2) when compared to kit 2 which had antibody titres above the detectable level (Figure 8.3).

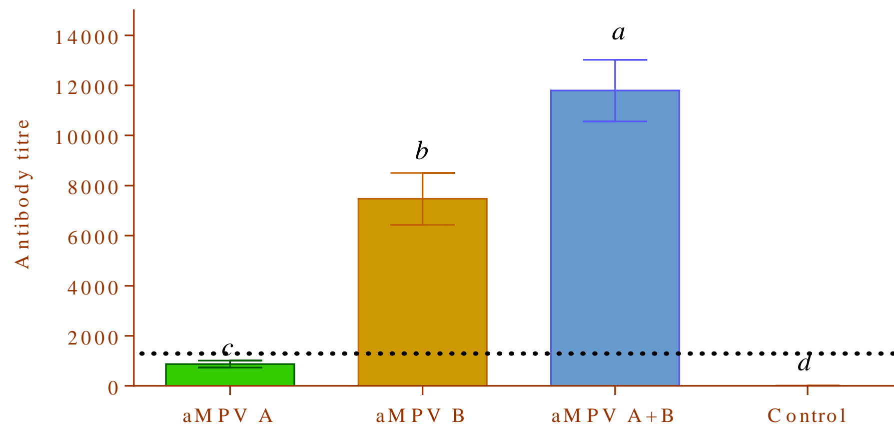

Figure 8.2. Kit 1. Mean of antibody titres against aMPV at 14dpi. Data presented as SEM (n= 10). Different letters demonstrate significant differences in response (P< 0.05). Dash line indicate the cut-off point = 1656.

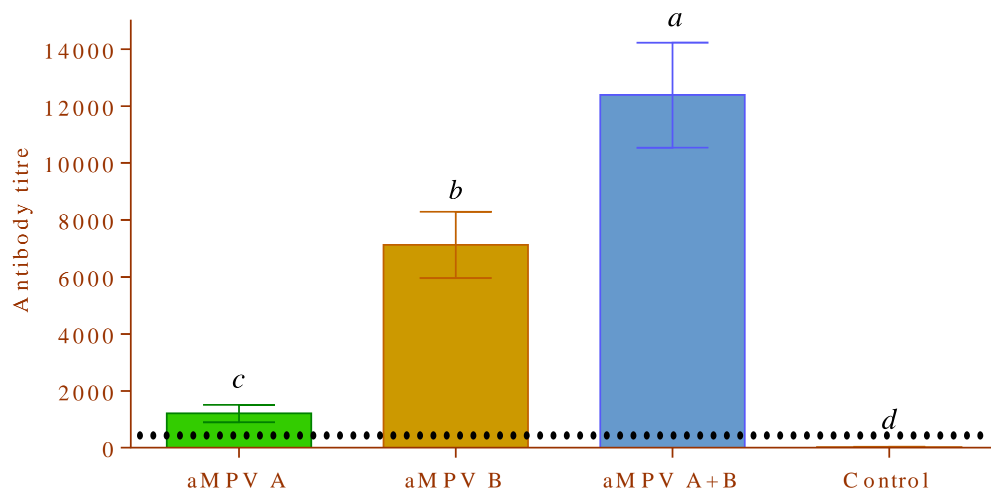

Figure 8.3. Kit 2. Mean of antibody titres against aMPV at 14dpi. Data presented as SEM (n= 10). Different letters demonstrate significant differences in response (P< 0.05). The cut-off titre= 396.

8.6. Discussion

Nasal exudates ranging from clear to turbid were the only clinical signs observed during this experiment. This correlates with previous findings that experimental infection of chickens with aMPV produces very mild clinical signs (Catelli et al., 1998; Nakamura et al., 1998). In this study, subtype A infected birds showed less severe and delayed onset of clinical signs when compared to birds infected with subtype B alone or in the co-infection of A+B. A previous study in broiler chicks also demonstrated that subtype A infected birds showed less severe clinical signs than those infected with subtype B (Aung et al., 2008). In another study, reported that a higher percentage of turkeys exhibited clinical signs after subtype B inoculation than of the subtype A (Liman and Rautenschlein, 2007).

In this study, up to the peak of clinical signs, the scores were significantly higher and more prolonged in the group given subtype B either singly or dually compared to the group that received only subtype A. There were significant differences in the clinical signs observed between birds infected with subtype B alone and those with co-infection. This suggests that the clinical severity was primarily contributed by the subtype B, as the initial virus inocula were virtually the same. Such enhanced disease severity was limited to the early phase of the infection, as at and after the peak of the clinical signs, there were no significant differences between these groups. It has been reported that different aMPV strains may display differences in virulence in chickens and turkeys (Cook et al., 1993; Liman and Rautenschlein, 2007) and in this study, a substantial differences were observed between the strains of aMPV subtype A and subtype B. We used aMPV subtype isolates from our laboratory and the results produced inevitably reflect the virulence of these isolates.

Globally, as each of aMPV isolate (either subtype A or B) may differ in its virulence, the findings reported here may not be representative of all or most A and B subtypes.

In term of aMPV detection in swabs or tissues, previous studies have shown that neither virulent subtype A or B were detected at or after 14 dpi in commercial broiler chicks (Aung et al., 2008; Rautenschlein et al., 2011). However, in this study aMPV B was detected in the turbinate, lung and spleen up to 14 dpi. Our findings are consistent with another study with aMPV subtype C, where the viral genome was detected in blood and lungs of infected chickens up to 15 dpi (Shin et al., 2000a). It is likely that in addition to the virus isolates used, the type of birds used may have influenced the outcome. Similar to our study, others have infected turkey poults with subtypes A and B and the viral genome of both subtypes were detected in the nasal turbinate for up to 14 dpi (Liman and Rautenschlein, 2007).

During the course of this study, we also found that birds infected with aMPV subtype B, either singly or as part of a co-infection with subtype A, were aMPV B positive for a longer period than in birds infected with subtype A alone. Subtype B has been shown higher virulence, a wider tissue distribution pattern and longer persistence than subtype A (Aung et al., 2008). In addition, aMPV was consistently detected in OP swabs and turbinate. This suggests that in chicks, for regular diagnostic and research purposes, OP swabs and turbinate could be the preferred samples of choice for aMPV detection. Using TOCs, subtype B was re-isolated from the turbinate up to 7 dpi and from the trachea for up to 5dpi. Previous studies have reported re-isolation of virulent aMPV B from nasal and sinus tissues, trachea and lungs of experimentally infected broilers up to 5 dpi and the virus was rarely

recovered thereafter (Catelli et al., 1998; Gharaibeh and Shamoun, 2012). Overall, there was poor isolation of subtype A as it was only isolated from the OP swabs at 3 dpi. Moreover, we were unable to detect subtype A in birds co-infected with both subtypes either by RT-PCR or VI. It is possible that virulence of this virus may have played an important role, where it was quickly eliminated by the host immune responses.

Birds infected with both subtypes together showed significantly higher levels of the antibody titre when compared to subtype B only infected birds as measured by both ELISA kits. In this study, with kit 1 low or no antibodies were detected in the subtype A infected chicks. In comparison, 80% of the same sera were positive for aMPV antibodies when the ELISA kit 2 was used. The choice of the coating antigen may have influenced the outcome, as the sensitivity of ELISA to detect antibodies varies with the kit and the coating antigen used (Eterradossi et al., 1995; Mekkes and de Wit, 1998). Previous studies have demonstrated that post infection sero-conversion induced by the French and UK aMPV isolates are significantly better detected if an antigenically related ELISA antigens were used (Eterradossi et al., 1992; Toquin et al., 1996). It appears that for a conclusive diagnosis of aMPV, appropriate ELISA kits should be used to screen the flocks and should be supplemented with molecular detection of the field aMPV subtypes.

Chapter 9: Evaluation of Flinders Technology Associates cards for storage and molecular detection of avian metapneumoviruses

9.1. Abstract

The feasibility of using FTA cards for the molecular detection of aMPV by RT-PCR was investigated. Findings showed that no VI was possible from aMPV-inoculated FTA cards, confirming viral inactivation upon contact with the cards. The detection limits of aMPV from the FTA card and TOC medium were $10^{1.5}$ CD_{50}/ml and $10^{0.75}$ CD_{50}/ml respectively. It was possible to perform molecular characterization of both subtypes A and B aMPV using inoculated FTA cards stored for up to 60 days at 4-6°C. Tissues of turbinate, trachea and lung of aMPV-infected chicks that were sampled either by direct tissue impression smears or inoculation of the respective tissue supernatants onto the FTA cards were positive by RT-PCR. However, the latter technique yielded more detection. FTA cards are suitable for collecting and transporting aMPV-positive samples, providing a reliable and hazard-free source of RNA for molecular characterization.

9.2. Introduction

RT-PCR has been established for detection of aMPV (Cavanagh et al., 1999). However, in some countries, due to lack of RT-PCR technology, expertise and economic factors, it is expedient to send the specimens for diagnosis to specialised laboratories in countries where expertise exists. Shipments of infectious material must comply with strict regulation in most of these countries and organisms must be inactivated by chemicals, such as phenol or formalin, before being transported (Snyder, 2002). However, chemically inactivated samples might not always prove efficient in terms of subsequently attempting to detecting the virus, due to problems in nucleic acid extraction (Coombs et al., 1999). An alternative and safe form of transportation of infectious material is the use of FTA cards (Moscoso et al., 2004). FTA cards comprise cotton-based cellulose paper which when treated with anionic detergents and buffer, provides a stable matrix for the immobilisation of genome for molecular characterization but free from the living host cells or organisms (Natarajan et al., 2000; Whatman, 2009). This technology simplifies the method of genome collection, storage, transportation, extraction, and consequently reduces the cost and time required (Mbogori et al., 2006).

The ability to use the FTA card for detection of several avian pathogens has been shown previously including *Mycoplasma* (Moscoso et al., 2004), IBV (Moscoso et al., 2005), NDV (Perozo et al., 2006), IBDV (Moscoso et al., 2006; Purvis et al., 2006), FAdV (Moscoso et al., 2007), Marek’s disease (MD) (Cortes et al., 2009) and AIV (Abdelwhab et al., 2011). To date, no information has been published on the use of FTA cards for detection of aMPV. In this study, using aMPV subtypes A and B, we investigated; the use of the FTA cards in inactivating aMPV (Experiment 1),

validating the detection limits for aMPV-inoculated FTA cards (Experiment 2), the effects of different storage temperatures on the detections of aMPV (Experiment 3). We also compared aMPV detections using two sampling techniques: tissue impression smears directly onto the FTA cards and same tissue macerated, and subjected to a freeze-thaw process and the supernatant inoculated onto FTA card (Experiment 4).

9.3. Materials and methods

9.3.1. Viruses

aMPV subtypes A and B were used in this study (Chapter 8.3.2).

9.3.2. FTA cards

FTA cards are a commercial product developed by the Whatman Corporation and are designed to transport and store a variety of biological materials including viral and bacteriological samples and blood (Figure 9.1). Infectious material applied to the cards is inactivated on contact as the cells are lysed but the genome is preserved for molecular recognition (Whatman, 2009).

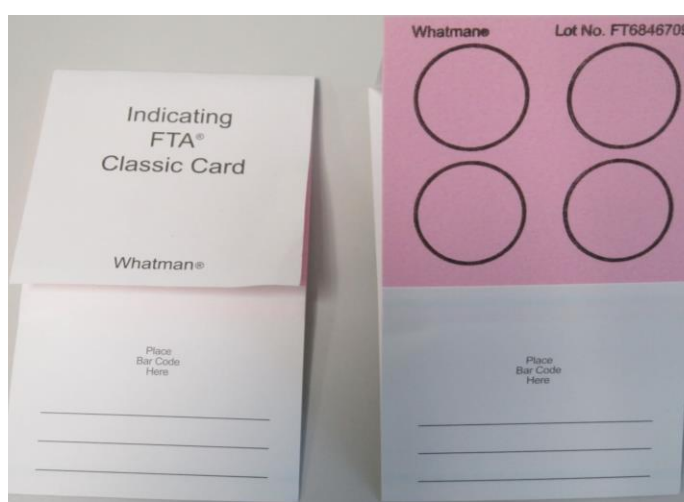

Figure 9.1. Flinders Technology Associates (Whatman Card).

9.4. Experimental design

9.4.1. Experiment 1. Confirmation of aMPV inactivation by FTA cards

One hundred microliters of stock culture of aMPV subtype A or subtype B were spotted onto the matrix area of an FTA card and allowed to air-dry at RT for 1hour. The spotted areas of the FTA card were cut out using sterile scissor and forceps. Each sample was placed into a bijou bottle containing 2 ml of TOC medium and processed for VI (Chapter 2.8.ii).

9.4.2. Experiment 2. Detection limits of aMPV on FTA cards

To determine the sensitivity of the RT-PCR in detecting subtype A or subtype B, serial 10-fold dilutions up to 10^{-7} were made from each subtype from the initial stock (Figure 9.2, A). For the each dilution, 100 µl was applied onto the matrix areas of the FTA cards which were air-dried for an hour at RT and kept away from direct light. For comparison, 300 µl of the same viral dilutions were subjected directly to RT-PCR. The RT-PCR reactions were run for each sample to determine the lowest dilution where viral RNA can be detected (Chapter 2.10.iii).

9.4.3. Experiment 3. Effects of storage temperature on detection of aMPV on FTA card

A volume of 100 µl of stock culture of aMPV subtype A or B was spotted onto the matrix circles of FTA cards. After air-drying as above, the cards were placed in sealed polythene bags and kept at three different temperatures, i) in a refrigerator, with temperature range of 4-6 ^{o}C, ii) in an incubator at 25°C, or iii) in an incubator at 41°C. On each of the following days: 1, 5, 10, 15, 20, 25, 30, 40, 50 and 60, one of the FTA cards was removed for the detection of aMPV by RT-PCR.

9.4.4. Experiment 4. Detection of aMPV subtypes from FTA cards *versus* tissue supernatants

To assess the different methods of sample submissions and to demonstrate any variations in aMPV detection, groups that infected with aMPV subtype A, subtype B and control birds (Chapter 8.4) were used in this experiment. At 1, 3, 5, 7, 9 and 14 dpi, samples of turbinate, trachea and lung were collected from five birds in each group.

The five tissues were each applied directly onto FTA cards. The same type of tissues was pooled. An impression smear was made by gently pressing the tissues onto the matrix area of the cards (Figure 9.2, B-C). The cards were labelled and air-dried but protected from direct sun light for an hour at RT. The remaining tissues were pooled according to the type, group and day of sampling (Figure 9.2, D) and stored at -70 ^{o}C until further use.

The same type of tissues was pooled and was manually macerated with sterile sand in a pestle and mortar, and 3 ml of TOC medium. After three cycles of freeze-thawing and centrifugation, 100 µl of each supernatant was spotted directly onto an FTA card and allowed to dry for 1 hour at RT. The FTA cards prepared with tissue impression smear and those which were inoculated with processed tissue supernatants were all examined for aMPV RNA.

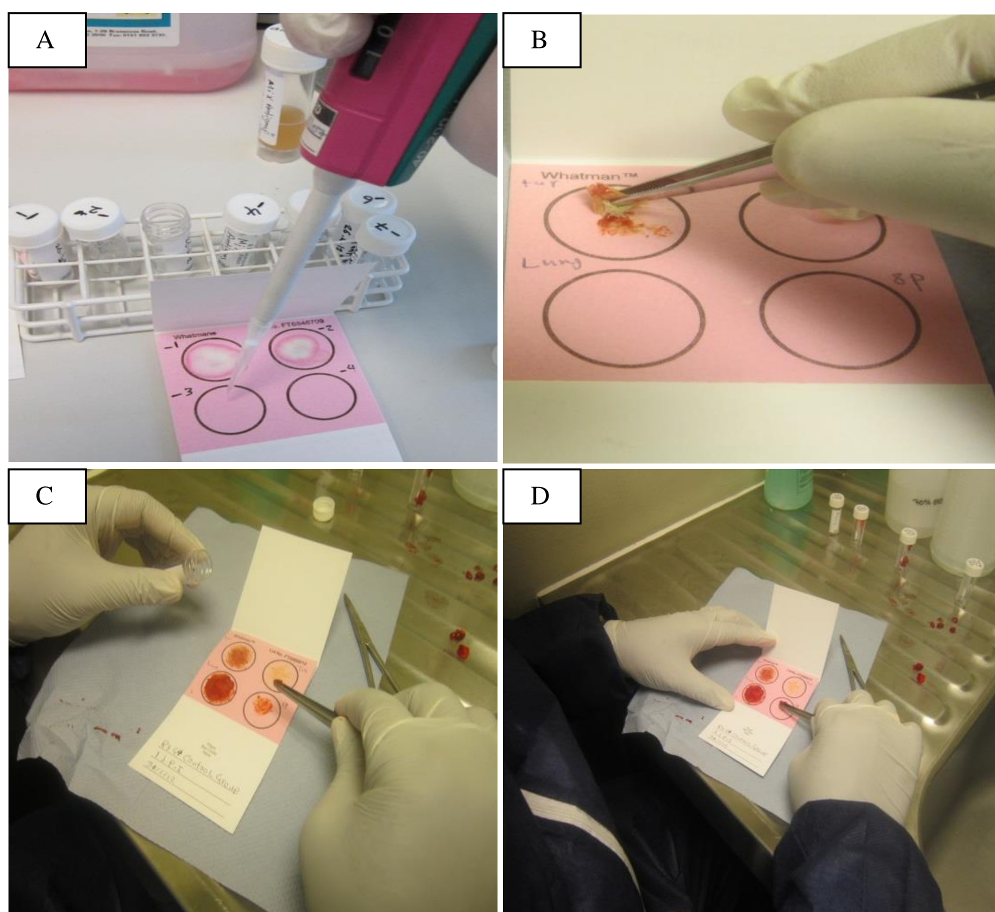

Figure 9.2. Shows application of tissue or medium into the FTA card.

9.4.5. Sample preparation for aMPV RT-PCR

Samples impregnated onto the matrix of the FTA cards (described above) and those with impression smear of fresh tissues were cut using sterile scissors and forceps and placed in bijou bottles containing 2ml of solution D then stored at -20°C. For extraction of the RNA from TOC medium, 300 µl was added to 300 µl of solution D. The RNA was extracted using phenol-chloroform method (Chapter 2.10.i) and RT-PCR was carried out as described in Chapter 2.10.iii.

9.5. Results

9.5.1. Experiment 1. Confirmation of aMPV inactivation by FTA cards

No ciliostasis was seen in the TOCs inoculated with elutions from either aMPV subtype-inoculated matrix of FTA cards. In contrast, in TOC rings that received the stock culture of subtype A or subtype B, ciliostasis occurred within 5-7dpi.

9.5.2. Experiment 2. Detection limits of aMPV on FTA cards

The detection limits by RT-PCR of the subtype A and subtype B aMPVs, based on either serially diluted stock cultures in the TOC medium or similar levels onto matrix of the FTA cards are shown in Table 9.1. Detection limits for subtypes A and B were $10^{1.56}$ CD_{50}/ml and $10^{1.51}$ CD_{50}/ml respectively for the samples processed from on the FTA cards. The detection limits of the subtypes processed from TOC medium were one log better than the FTA cards.

Table 9.1. Detection limits of aMPV subtype A or B on FTA cards.

Virus dilution	Initial stock of aMPV A ($10^{4.56}$ CD_{50}/ml) and aMPV B ($10^{4.51}$ CD_{50}/ml)			
	FTA card*		TOC medium≠	
	aMPV A	aMPV B	aMPV A	aMPV B
10^{0}	+	+	+	+
10^{-1}	+	+	+	+
10^{-2}	+	+	+	+
10^{-3}	+	+	+	+
10^{-4}	-	-	+	+
10^{-5}	-	-	-	-
10^{-6}	-	-	-	-
10^{-7}	-	-	-	-

**Hundred microliters of serial dilutions up to 10^{-7} applied into the circles present in the FTA cards; ≠Three hundred microliters of serial dilutions up to 10^{-7} from TOC medium which contains the virus was used directly to RT-PCR.*

9.5.3. Experiment 3. Effects of storage temperature on detection of aMPV on FTA cards

The stability of viral genome on FTA cards was measured by performing RT-PCR on the stock virus spotted onto FTA cards and stored at different temperatures for up to two months. The viral genome of both subtypes A and B was detected when stored at 4°C for up to 60 days post storage (dps). Subtype A RNA was detected only up to 1 dps at 25 °C and 41°C. In contrast, subtype B RNA was detected up to 30 dps at 25°C and for up to 5 dps for when stored at 41°C (Table 9.2).

Table 9.2. Stability of aMPV RNA on the FTA cards under differing storage temperatures.

dps	**aMPV A***			**aMPV B***		
	4°C	**25°C**	**41°C**	**4°C**	**25°C**	**41°C**
1	+	+	+	+	+	+
5	+	-	-	+	+	+
10	+	-	-	+	+	-
15	+	-	-	+	+	-
20	+	-	-	+	+	-
25	+	-	-	+	+	-
30	+	-	-	+	+	-
40	+	-	-	+	-	-
50	+	-	-	+	-	-
60	+	-	-	+	-	-

* *Hundred microliter of the initial stock of the aMPV subtype A or B were spotted on the active area of the FTA and stored at three different temperatures.*

9.5.4. Experiment 4. Detection of aMPV subtypes from FTA cards *versus* tissue supernatants

No virus was detected in the control birds. Table 9.3 shows the results for the infected groups. For subtype A, there were less frequent detections than subtype B. Subtype A was detected up to 7 dpi only, compared to subtype B, which was detectable up to 14 dpi (end of the experiment). In comparison of the tissue impression smears and supernatants, for subtype A, there were marginally more frequent detections in the tissue impression smears. In contrast, for the subtype B, there were no obvious differences with detection in the turbinates with either method of sampling; virus was detected at almost at all sampling intervals, except at 5 dpi where the virus was detected only in the tissue impression sample. For tracheal samples, there was obvious difference between the methods but for lungs there was slightly longer detection by tissue impression methods (up to 9 dpi). Overall, for subtype A and B infections, by either sampling methods (impression smear or tissue supernatant), the greatest level of detection was seen at 5 dpi.

Table 9.3. A comparison of the detection of aMPV by RT-PCR on samples collected on FTA cards either as tissue impression smear or inoculated with tissue supernatant.

Tissue	Groups	Methods of tissue sampling onto FTA card	dpi					
			1	3	5	7	9	14
Turbinate	A	Impression*	-	+	+	+	-	-
		Supernatant ≠	+	+	+	-	-	-
	B	Impression	+	+	+	+	+	+
		Supernatant	+	+	-	+	+	+
Trachea	A	Impression	-	-	+	+	-	-
		Supernatant	-	+	+	-	-	-
	B	Impression	-	-	+	-	-	-
		Supernatant	+	+	+	-	-	+
Lung	A	Impression	-	+	+	-	-	-
		Supernatant	-	-	+	-	-	-
	B	Impression	-	-	+	+	+	-
		Supernatant	+	-	+	+	-	-

*****imprint was made by gently pressing the tissue against providing matrix area of the FTA card; ≠One hundred microliters of the tissue supernatant that had undergone three times of freeze-thaw was spotted directly onto FTA card.*

9.6. Discussion

Definitive diagnosis of aMPV infection requires identification of the virus in clinical material which is most often achieved nowadays by RT-PCR. However, PCR technology is unavailable in some countries and requires transportation of samples in a safe way to specialist laboratories, with international shipment of samples needing high standards of biosafety procedures (Snyder, 2002), including for aMPV. In assessing the suitability of detecting aMPV subtype A or B, or both, we are reporting the results of experiments on inactivation of aMPV on FTA card, aMPV detection limits when the viruses are sampled on FTA cards, effects of different temperatures on storage of FTA cards containing aMPV, and a cross-comparison between two sampling methods (direct impression smear versus inoculation of tissue supernatant onto FTA cards).

The absence of ciliostasis of the TOCs inoculated with elution from aMPV subtype A or subtype B indicated no viable virus presence, confirming that the FTA card inactivates the aMPV viruses. The ability of FTA cards to inactivate other avian pathogens such as IBV, NDV, IBDV, AIV and FAdV have been reported before (Moscoso et al., 2005; Moscoso et al., 2006; Perozo et al., 2006; Moscoso et al., 2007; Narayanan et al., 2010; Abdelwhab et al., 2011).

In our hands for samples on FTA cards, the detection limits for subtype A and subtype B were $10^{1.56}$ CD_{50}/ml and $10^{1.51}$ CD_{50}/ml respectively. These values were approximately one log lower than the detection limits of viruses in the TOC medium. These data are in agreement with similar study on detection of multiple poultry respiratory pathogens on FTA cards (Awad et al., 2012). It appears that there could be a small loss of RNA in the process of inoculation onto the FTA card matrix,

storage or extraction processes. However, such loss would out-weight the benefit of aMPV detection, particularly for samples transported from thousands of miles.

Ideally, for successful virus detection, the FTA card should preserve and protect against deteriorations of viral genome. The FTA card manufacturer states that the card could be used for sampling and transportation of RNA viruses and the FTA card can be stored for a short term at RT or longer at -20°C or -70°C (Whatman, 2009). However, to date, there is no published information of the effects of various environmental temperatures on the detection of aMPV by RT-PCR. Our study demonstrated that following inoculation of FTA cards and storage in air-tight plastics bags at 4-6 ^{o}C subtypes A and subtype B were both detected for up to 60 days. Interestingly, at 25 ^{o}C, aMPV subtype B was detected up to 30 days but only for 1 day for the subtype A. The reason for this discrepancy is unclear even though virus titres of the viruses were almost the same. When the FTA card were stored at 41°C, mimicking summer temperature in some countries, the subtype A was again only detected for one day but the subtype B was detected up to 5 dps. This reflects poor preservation of aMPV RNA at this temperature. The decrease in the sensitivity could be due to that RNA denatures over time and is faster at higher temperatures (Rogers and Burgoyne, 2000; Moscoso et al., 2005; Perozo et al., 2006). As such, FTA cards intended for aMPV RT-PCR, ideally need to be stored at low temperature as possible, preferably 4-6 ^{o}C. RT-PCR detection of IBV or IBDV in samples stored on FTA cards at -20°C, 4 ^{o}C or 41 ^{o}C indicates that the RNA was stable for at least 15 days (Moscoso et al., 2005; Moscoso et al., 2006). NDV was stable on the FTA card for at least 20 days at both RT and 4°C and for 18 months at -20°C (Narayanan et al., 2010). AIV RNA was also stable on FTA cards for at least five months at RT (Abdelwhab et al., 2011). It appears that other than the intrinsic

nature of the viruses, the variations in the virus titres and differences in the PCR protocols may have influenced the outcome.

Experiment 4 was organised to answer field questions on the appropriate methods of samplings onto FTA cards for optimal detection. The question was whether it was better to do a tissue impression smear directly onto the FTA card or should the tissues be processed in the normal manner for VI and freeze-thawed three times, before applying a drop of 100 µl onto the FTA cards. Our study showed that there was slightly better chances of detecting aMPV RNA when the sample supernatant were used compared to tissue impression smears. These findings suggest that with tissue impression smears there could be a reduced amount of RNA left on the FTA card. In contrast, for the tissue supernatant, it is likely that maceration and later freeze-thaw processes have allowed cells to rupture and release of more viral RNA into the supernatant. In a study with AIV RNA from swab samples from the field adsorbed to and extracted from FTA cards was detected with reduced sensitivity when compared to RNA directly extracted from swab fluids (Abdelwhab et al., 2011).Thus, it appears that to increase the chances of aMPV detection, it is worthwhile to grind the tissues (a pool of five or more tissues), subjected them to a freeze-thaw process, centrifuge (or allow to stand-still) and apply the supernatant onto the FTA cards.

These findings showed that the virus could be detected on FTA card samples taken from the turbinate, trachea and lung. However, the length of time for which detection was possible varied according to the organ type, sampling time and variation between aMPV subtypes A and B. For example, with samples taken from the turbinate, aMPV subtype A and B were detectable up to 7 and 14 dpi respectively, and with those from the lung were positive up to 5 and 9 dpi for aMPV A and B

respectively. These indicate that subtype B virus can be detected for a longer period than subtype A (Aung et al., 2008), even though, similar titres of the viruses were used. The highest detections of aMPV was recorded at 5 dpi and this is in agreement with previous work in broilers (Gharaibeh and Shamoun, 2012) and turkeys (Liman and Rautenschlein, 2007).

We conclude that FTA cards are suitable for collecting and transporting aMPV samples worldwide but the cards must be stored at lower temperatures for optimal chances of aMPV detection.

Chapter 10: General discussion and future work

This chapter outlines the salient features of the experimental results described previously. Various aspects of the immunopathogenesis, molecular diagnosis and vaccination programmes of IBV and aMPV were examined in these experiments and on the basis of our findings some possible future work is discussed.

The constant emergence of variant strains of IBV poses a serious threat to vaccination strategies. One of the most prominent IBV strains in the Middle East and North Africa designated as IS/885/00 remains a major threat to the poultry industry since first reported in Israel in 2000 (Meir et al., 2004). Very little information is available in the literature regarding the manifestations of the disease caused by this variant strain of IBV. Hence, a study was conducted on the immunopathogenesis of this strain in one-day old SPF and IBV MDA positive broiler chicks (Chapter 3). The IS/885 strain was found to be pathogenic to both types of birds. An important finding in this study was one broiler bird suffering from mild head swelling. Samples taken post mortem and subjected to VI and RT-PCR were found to be positive for IBV. On sequencing the virus isolated was found to be 100% similar to original challenge virus (IS/885). Though, IBV M41 has been associated with SHS in chicks (Droual and Woolcock, 1994), this appears to be the first report on development of head swelling following IBV infection under experimental condition.

In addition, the characteristic cystic oviduct previous reported for IBV strain M41 (Crinion et al., 1971; Crinion and Hofstad, 1972a) and QX (Benyeda et al., 2009; de Wit et al., 2011b; Ganapathy et al., 2012) infections was observed in two female SPF chicks infected with IS/885. IBV isolated from the cystic fluid showed, on sequencing of the part-S1 gene a 99% similarity to the original IS/885 inoculum. As a result of our findings, further work is needed to understand the pathogenesis of this

variant IBV on the development of cystic oviducts and subsequent effects on egg production and quality.

It has been shown that the conventional Mass vaccines alone do not provide sufficient protection against IS/885/00 and IS/1494/06 (Meir et al., 2004; Kahya et al., 2013). Thus, the study in Chapter 4 was designed to evaluate the protection induced by commercial live IBV vaccines in day-old commercial broiler chicks against challenge with these strains. It appears that a combination of live H120 and CR88 vaccines given at day-old followed by CR88 vaccine at day 14 of age confer an excellent protection against both variant IS/885 and IS/1494 viruses. The H120 at day-old followed by CR88 at 14 days-old, provided somewhat lower protection, in particular against IS/885. Further work is required to understand the underlying immune mechanisms for such higher and broader of protection conferred by these distant vaccine viruses.

Relatively little research exists regarding cellular and local immune responses induced by IBV vaccination. To this end, the role of humoral, cellular and local immunity induced by administration of different live IBV vaccines in one-day old IBV MDA positive broiler chicks were studied in Chapter 5. It was observed that CD4+ cells were recruited into the trachea earlier than CD8+. This observation was in accordance with a previous study, which observed that CD4+ cells outnumbered CD8+ cell in the trachea (Janse et al., 1994). In contrast, Dhinakar Raj and Jones (1996a) reported that CD8+ cells were recruited into the trachea earlier than CD4+ cells after infection with virulent 793B. Based on these conflicting results further investigations are required. One possible explanation for this anomaly could be due to differences in IBV strains.

To our knowledge, there has been no information available regarding circulating IBV variant in Libya. In June 2012, swabs and tissue samples were collected from diseased broiler flocks experiencing high mortality and respiratory distress (Chapter 6). IBVs with high similarity to regionally important strains IS/885/00-like and IS/1494/06-like were detected by RT-PCR. Further studies on the spread of IBV in Middle Eastern countries and North Africa should include the mode of spread, isolation and serotyping/genotyping of IBVs in the region.

Previous studies (Cook et al., 2001b; Ganapathy et al., 2005a; Tarpey et al., 2007) suggested that CMI may have played an important role in conferring the protection against challenge viruses of NDV, aMPV or IBV. In Chapter 7, we evaluated the cell-mediated and local immune responses in the trachea of birds following administration of single, dual or triple combinations of live NDV, aMPV and IBV vaccines using IHC. In the vaccinated groups a significant increase was observed in the expression of CD4+, CD8+ and IgA-bearing B cells in the trachea when compared to the unvaccinated group. However, within the vaccinated groups, the levels of expression of each of the cells did not differ significantly. These observations demonstrate the role of the CMI response following NDV, aMPV and IBV vaccination in chickens.

In Chapter 8, even though similar titres of the viruses were used, the result demonstrated that aMPV subtype B was more pathogenic than subtype A. Our findings are in agreement with those of Aung et al. (2008) who observed that in a subtype B infected group, higher numbers of broilers showed clinical signs, a wider tissue distribution and a longer period of aMPV-positive samples than in subtype A infected group. Following on from these findings it would be interesting in the future

to conduct further studies encompassing other aMPV subtypes A and B isolated from different region to see if similar results are found.

In chapter 9 a diagnostic study using FTA cards for the molecular detection of aMPV by RT-PCR was investigated. One of the key findings showed that at 25 ^{o}C aMPV subtype B was detected up to 30 dps but only for 1 dps for the subtype A. The reason for this discrepancy is unclear and it needs further investigation.

In summary, the studies undertaken for this project have provided some useful practical insights in the pathogenicity, immunity, diagnosis and control of IBV and aMPV infection in chickens. Our results have been published in scientific journal and presented in national and international meetings.

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Appendix 1

(Reagents and protocols)

Stock solution D

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

Kept for one month at 4°C

Working solution D

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

TOC culture medium and supplements

10x Minimum Essential Medium-Eagles [41]	100 ml
7.5% sodium bicarbonate [40]	20 ml
Sterile distilled water	900 ml
Penicillin-streptomycin solution	4 ml

Stock antibiotic solution

Crystapen[42]	600 mg
Streptomycin sulphate BP [43]	1.0 g
Sterile distilled water	4 ml

[40] Sigma-Aldrich, UK

[41] Life Technology, UK

[42] Glaxo, Greenford, UK

[43] Evans Medical, Greenford, UK

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.

HEPES buffer

HEPES [44]	2.95 gm
NaCI[45]	4.09 g
$CaCl_2$ [45]	0.08 g

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

Phosphate buffered saline (PBS)

NaCl	85.00 g
Di-sodium hydrogen orthophosphate (Na_2HPO_4)	10.70 g
Sodium dihydrogen orthophosphate ($NaH_2PO4.2H_2O$)	3.90 g

Made up to 1 litre with sterile distilled water

2M sodium acetate

Glacial acetic acid[46]	19.00 ml
Sodium acetate[47]	5.46 g

Made up to 200 ml with sterile distilled water

[44] Life Technology, UK

[45] Sigma-Aldrich, UK

[46] VWR International Ltd

[47] Sigma-Aldrich, UK

0.75M Sodium citrate pH7.0

Citric Acid Monohydrate	31.52 g
NaOH pellets	16.8 g
Sterile distilled water	200 ml

Resuspending (RS) water

RNasin, RNAse inhibitor [48]	2.5 µl
Dithiothreitol (DTT) [49]	5 µl
Ultra-pure water [47]	92.5 µl

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

IBV Oligos[50]

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

[48] Promega, Madison,USA

[49] Invitrogen, Paisley, Scotland

[50] Invitrogen, Paisley, Scotland

aMPV Oligos [50]

Working Oligo	Stock Oligo		Sigma Water
G6-	10 µl		90 µl
G5-	10 µl		90 µl
G8+A	10 µl		90 µl
G9+B	10 µl		90 µl
	A	**B**	
G1+	10 µl	10 µl	80 µl

NDV Oligos [51]

Working Oligo	Stock Oligo	Sigma Water
MFS+	10 µl	90 µl
MSF-	10 µl	90 µl

10mM dNTP's Working Solution

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

Take 20µl of each dNTP, total volume 80µl

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

[51] Invitrogen, Paisley, Scotland

RT-PCR reaction for IBV/aMPV

5X Buffer [51]	1 µl
DTT	0.5 µl
dNTP [51]	0.25 µl
Ultra-pure Water	2.13 µl
RNase inhibitor	0.12 µl
Superscript II (200 µ/ µl) [51]	0.25 µl
Negative Oligo (10 pmoles/µl) (IBV SX2-) (aMPVG6-) (MFS-)	0.75 µl

Nested PCR 1 protocol

PCR Supermix [52]	19 µl
+ Oligo (IBV SX1+) (aMPV G1+) (MSF-)	0.5 µl
- Oligo (IBV SX 2-) (aMPV G6-) (MSF+)	0.5 µl

Nested PCR 2 protocol

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

Loading Buffer

Ficol	3 g
1XTBE	20 ml

[52] Invitrogen, Paisley, Scotland

10x Tris-borate-EDTA (TBE)[53]

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

Gel preparation

1.5% agarose [53]	0.58 g
TBE	35 ml

[53] Sigma-Aldrich, UK

QIAamp viral RNA extraction

Equilibrate samples and buffers to RT

Addition of carrier RNA to buffer AVL

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

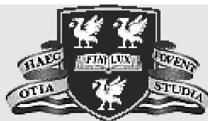
Protocol

1. Add 560 µl of prepared buffer AVL containing reconstituted carrier RNA into 1.5 ml eppendorf tubes
2. Add 140 µl of sample
3. Vortex for 15sec
4. Incubate at RT for 10min
5. Pulse centrifuge
6. Add 560 µl of 100% ethanol
7. Vortex for 15sec

8. Pulse centrifuge
9. Label spin columns
10. Carefully remove 630µl from tube (step 6) into spin column
11. Centrifuge at 8000 rpm for 1minute, discard flow through
12. Place spin column in clean collection tube and repeat steps 10 and 11
13. Place spin column in clean collection tube add 500 µl of buffer AW1
14. Centrifuge at 8000 rpm for 1min, discard flow through
15. Place spin column in clean collection tube add 500 µl of buffer AW2
16. Centrifuge at 13,000 rpm for 3minutes, discard flow through
17. Place spin column in clean 1.5 ml eppendorf tube add 60 µl of sigma water
18. Incubate at RT for 1 minute
19. Centrifuge at 8000 rpm for 1min, discard spin column and save flow through (RNA)
20. Store RNA at -20°C

Appendix 2

(Data collection sheets)



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Result sheet for ciliostasis test

Group................

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

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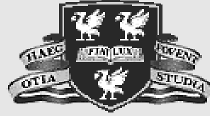
Result sheet for IBV clinical signs

Group.................

Date	Day	dpi	Comments



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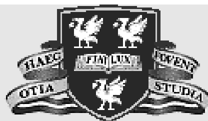
Result sheet for gross lesions

dpi ....

Bird No	Control		IS/885		IS/1494	
	Trachea	**Kidney**	**Trachea**	**Kidney**	**Trachea**	**Kidney**
1						
2						
3						
4						
5						



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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												

Date:

Name:

Project:

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Result sheet for IHC data

Group ....

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

Appendix 3

(Published manuscripts)