Significance of Viral Subpopulations within Arkansas Serotype Infectious Bronchitis Virus Vaccines

by

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Abstract

As a result of spontaneous mutations and/or recombination occurring within the viral genome, infectious bronchitis virus (IBV) exists as multiple serotypes and genotypes that poorly cross protect. Molecular analysis of many field isolates in the United States (US) has revealed that the most frequent IBV isolates are Arkansas (Ark) DPI isolates despite extensive use of Ark DPI derived vaccines in the field. The factors behind the predominance of Ark DPI IBV isolates are unclear. Analysis of ArkDPI derived vaccines currently used in the US revealed that they are heterogeneous and that some minor viral subpopulations within these vaccines efficiently replicate in the upper respiratory tract of chickens and are selected as early as three days post vaccination. The proportion of these minor subpopulation differed among the Ark vaccines.

We analyzed the significance of these viral subpopulations within the Ark serotype vaccines. We found that Ark vaccines containing high proportions of the selected subpopulations resulted in higher viral loads, more severe respiratory signs and tracheal pathology but also a higher immune response. In addition we found that temporal evolution occurs after Ark vaccine administration, resulting in some of the minor subpopulations persisting longer in the respiratory tract than others. These subpopulations

were also more readily transmitted to contact non-vaccinated birds than others, further suggesting that these subpopulations have better fitness? When given simultaneously with IBV Mass serotype vaccine, we found interference with Ark vaccine virus replication as compared to when the Ark vaccines were administered alone, a factor that may contribute to poor immune response induction by some Ark vaccines. Persistence of Ark vaccine viruses may also give these viruses ample time to mutate and/or recombine and time for selection to occur resulting in generation of more virulent variants.

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CHAPTER 1

LITERATURE REVIEW

Introduction

Avian infectious bronchitis is a viral disease caused by a group 3 coronavirus, infectious bronchitis virus (IBV). The disease has been described as the single most economically important infectious diseases of chickens in regions where chickens are intensively reared. First described in the United States (US) in the 1930's, the disease affects all ages of chickens but clinical signs are more pronounced in the young (Schalk & Hawn, 1931). All breeds of chickens are susceptible although it is not clearly understood if the severity of clinical disease is uniform across the breeds (Cavanagh, 2007).

Infectious bronchitis virus exists as several different poorly cross protecting serotypes worldwide. This is due to the rapid and spontaneous mutations that occur within the hypervariable region of the envelope spike glycoprotein (S) gene, the protein responsible for inducing neutralizing and serotype specific antibodies.

Control of IBV has been through the use of live attenuated and inactivated vaccines usually of several serotypes. The live attenuated vaccines are the most commonly used in all types of poultry. The live attenuated vaccines offer good immune response but often require revaccination (Cavanagh & Gelb, 2008).

Inactivated vaccines are given to layers and breeders before lay as booster vaccines (reviewed in Cavanagh, 2007).

Use of live attenuated vaccines for control of viral diseases is well known to be effective. However, there are issues concerning the ability of some vaccine viruses to revert to virulence when given back to the host (Hopkins & Yoder, 1986; Nix *et al*., 2000). Re-circulation and persistence of vaccine strains in the environment may also be associated with emergence of new strains after recombination events (Wang *et al*., 1993; Jia *et al*., 1995). Although current vaccines have reduced disease prevalence rates, new cases of respiratory disease due to IBV in previously vaccinated flocks continue to be reported. Notably in the US southeast where most of the US poultry are reared, cases of IBV due to Arkansas (Ark DPI) type viruses (Nix *et al*., 2000; Toro *et al*., 2006) are the most common and also nationwide in the US (Jackwood *et al*., 2005). The factors behind this frequent isolation of ArkDPI type viruses are unknown. Jackwood *et al*. (2005) and Nix *et al*. (2000) analyzed Ark-like field isolates using spike (S1) gene reverse transcription polymerase chain reaction (RT-PCR), restriction fragment length polymorphism (RFLP) and sequencing and concluded that the Arkansas (ArkDPI) serotype vaccines being used might be a source of the ArkDPI viruses in the field. In addition Jackwood *et al.,* (2009) found that Ark vaccine viruses were persisting longer than other serotypes in multiple serotype vaccinated flocks. Nix *et al*. (2000) found viral subpopulations in two commercial IBV vaccines, original stock of attenuated ArkDPI distributed to vaccine companies and also the original virulent strain. We and others have found that four commercial IBV ArkDPI derived vaccines currently in use in the US contain minor viral subpopulations that are selected as early as 3 days post vaccination

DPV) (van Santen & Toro, 2008; McKinley *et al*., 2008; Gallardo *et al*., 2010). These subpopulations are apparently more fit to replicate in the upper respiratory tract of chickens than the major vaccine population. The proportions of these minor subpopulations differ among the different commercial Ark vaccines. In some vaccines, the selected subpopulation is the predominant subpopulation present, while in others it is only detectable by RT-PCR with primers targeting this subpopulation. We also found that the S1 subunit spike gene sequences of the most frequently selected subpopulations are more similar to the unattenuated ArkDPI vaccine parent than to the major vaccine population (van Santen & Toro, 2008). In a recent study, the various vaccine subpopulations were found in experimentally inoculated birds in different tissues at varying incidences indicating that they may also have different tissue tropisms (Gallardo *et al*., 2010).

Whether the presence of vaccine subpopulations contribute to the current predominance of IBV ArkDPI viruses is unclear. This dissertation reports a detailed evaluation of the significance of the presence of these subpopulations within Ark-DPI-derived Ark serotype vaccines currently in use in the US. The significance of the different vaccines' subpopulation structure in terms of induced respiratory signs, tracheal pathology, immunity, persistence in the host, transmission to contact non-vaccinated chickens and interaction with other IBV vaccine serotypes is analyzed and discussed. The broad goal is to understand the basis of increased isolation of IBV ArkDPI viruses in the southeastern US despite the extensive use of ArkDPI derived vaccines.

Overview of coronaviruses

Coronaviruses belong to the family *Coronaviridae*, in the order *Nidovirales*. These viruses are enveloped, can take many shapes and have large club-shaped surface projections from their envelopes that give them a 'corona' (crown)-like appearance hence their name. They are divided into three groups based on serologic and genetic relationships (Cavanagh, 2007; Lai *et al*., 2001; Gonzalez *et al*., 2003 and Weiss and Navas-Martin, 2005).

Coronaviruses have the largest genomes among RNA viruses, and range in size between 26-32 kilobases (Lai *et al.,* 2001). The genomes are single-stranded, positive sense, helical and complexed with the basic nucleocapsid protein (N). In general, the genomes of all coronaviruses have similar organization and code for four structural proteins spike (S), small envelope protein (E), membrane glycoprotein (M), and N in this order. Genes for the structural proteins occupy a third of the genome at the 3'end. S, E and M are membrane proteins found in all coronaviruses although group 2 has another membrane-associated protein, hemagglutinin-esterase, not found in other groups. Several other relatively smaller genes that encode non-structural proteins are found interspersed among the structural protein genes. The number and location of these genes differs among groups and species. The genome has two untranslated regions (UTRs) located on the 5' and 3' ends of the genome. In IBV, each of these regions is approximately 500 nucleotides in length (reviewed in Cavanagh 2007; Weiss & Navas-Martin 2005). An important non-structural gene, gene 1, is found in the 5' end of the genome and occupies two-thirds of the total genome. This gene codes for the replicase proteins ranging from 15-16 proteins in most coronaviruses. These proteins are translated by frameshift

mechanism from two large overlapping open reading frames (ORF1a and ORF1b) into two large polypeptides ppp1a and pp1b (reviewed in Weiss & Navas-Martin, 2005). These polyproteins are cleaved to their respective constituent proteins by viral proteases (reviewed in Gadlage & Denison, 2010).

Coronavirus S protein

The S protein, one of the most important coronavirus membrane glycoproteins, projects from the envelope in dimer or trimer form and appears as club shaped crown-like projections that give the family its name 'corona' (Lai, 1990). The protein has two main functions: attachment to host cell surface receptors and activation of fusion of the virion membrane with the host cell membranes during entry (Gallagher & Buchmeier, 2001; Bosch *et al.,* 2003). The S protein has two domains, the extracellular S1 domain, responsible for attachment of the virus to host cell and the trans-membrane S2 domain, which is involved in fusion of the viral and host cell membrane. In some coronaviruses, the S protein is cleaved into S1 and S2 subunits. In both IBV and murine hepatitis virus (MHV) the S protein is cleaved, while in some coronaviruses like transmissible gastroenteritis virus (TGEV) and the highly fatal feline infectious peritonitis virus (FIPV), it remains in an uncleaved form (Lai, 1990). In all coronaviruses, the amino-terminal S1 subunit is found externally, and the carboxy-terminal S2 subunit anchors the whole S protein onto the viral envelope (Casais *et al*., 2003; Cavanagh, 2005, 2007).

The S protein has been shown to be a determinant of host cell range in many coronaviruses. In coronaviruses infecting swine for example, analysis of recombinants of swine enteric and respiratory coronaviruses showed that loss of enteric tropism required change of two amino acids at the N terminus of spike protein (Ballesteros *et al*., 1997).

Furthermore, acquisition of enteric tropism and virulence was induced in a recombinant attenuated helper respiratory recombinant virus containing the TGEV S gene indicating that the S protein in porcine coronaviruses is both a cell tropism and virulence determinant (Sanchez *et al*., 1999). In addition, analysis of neutralizing-antibody-resistant mutants of TGEV revealed that point mutations occurring within the antigenic S1 region were associated with acuteness of infection in newborn piglets suggesting the spike protein role in pathogenicity (Bernard & Laude, 1995). Simultaneous substitution of two human severe acute respiratory syndrome (SARS) coronavirus spike protein amino acids 479 (N to K) and 487 (T to S) to those of a palm civet isolate abolished the human SARS coronavirus ability to infect cells expressing the human (angiotensin converting enzyme-2) ACE2 receptor and vice versa indicating involvement of spike in species tropism. Recombinant spike proteins containing any of these two individual amino acid substitutions did not completely abolish receptor interaction but the receptor affinity was reduced (Qu *et al*., 2005). The role of coronavirus S protein in determining host specificity, host cell range and also pathogenicity has also been shown for feline coronaviruses (Haijema *et al*., 2004; Rottier *et al*., 2005). Replacement of FIPV S protein with that of MHV by targeted recombination altered the host tropism resulting in feline virus (FIPV) ability to replicate in murine cells. The virus consequently lost ability to replicate in feline cells (Haijema *et al*., 2003). Through construction of hybrid viruses with genomes of both mild feline enteric coronaviruses (FECV) and highly virulent FIPV, ability to infect macrophages resulting in increased virulence was mapped to the FIPV S protein, specifically the carboxyl terminus (Rottier *et al*., 2005). For the murine coronavirus MHV, the S protein was also found to be a determinant of both cell tropism

and pathogenicity (Phillips *et al*., 1999; Navas *et al*., 2001). Recombinant viruses containing the S protein of A59, MHV-2, or MHV-4 in the A59 genome backbone were analyzed for their ability to replicate and cause hepatic lesions. Acquisition of the MHV-2 S protein gene made the recombinants able to replicate to high titers similar to wild type MHV-2 and cause liver pathology (Navas *et al*., 2001), while ability to infect and cause meningoencephalitis was seen in a MHV recombinant A59 containing the MHV-4 S gene (Phillips *et al*., 1999). Using reverse genetics technology, recombinant IBV containing the genome of Beaudette and ectodomain region of the S protein from the M41-CK, Casais *et al.,* (2003) demonstrated that S protein of IBV is a determinant of cell tropism. The recombinant IBV Beaudette acquired similar cell tropism as the M41-CK, the source of the S protein gene. Fang *et al*. (2005) also found that the majority of mutations occurring in the genome of IBV during adaptation to Vero cells occurred in the S gene. Evaluation of genetic changes during embryo and chicken passage of European and North African IBV serotype 793/B strain revealed that a single amino acid at position 95 of the S1 portion of the genome was the only notable change. The amino acid alternated between a serine and alanine between embryo and chicken-passaged virus (Cavanagh *et al*., 2005; Callison *et al*., 2001). Analyzing IBV vaccine viruses, van Santen & Toro, (2008) and McKinley *et al.,* (2008) found that IBV vaccine viruses selected in vaccinated chickens differed in their S1 gene sequences from the embryo passaged IBV vaccine viruses, suggesting that these S1 differences may be determinants for host cell tropism. In addition, IBV vaccine viral subpopulations found in different chicken tissues differed in their S1 gene sequences further revealing the role of S1 in determining cell tropism (Gallardo *et al*., 2010). In IBV although studies have revealed the role of S

protein in determining cell tropism, additional factors are thought to be involved in determination of virulence (Hodgson *et al*., 2004).

Other coronavirus proteins

The other coronavirus structural proteins include the M glycoprotein, a 230 amino acid and the most abundant virion protein (Weiss & Navas-Martin, 2005) and the slightly smaller E protein (100 amino acids). Both these proteins are thought to play a role during viral particle assembly (Vennema *et al*., 1996). In TGEV, the M protein is thought to play a role in inducing interferon alpha, an innate immune protein important for activating immune cells against viral infection (Laude *et al*., 1992) while the E protein is essential since its disruption is lethal to the virus. The E protein is also thought to play a role in production of infectious virus in MHV (Weiss & Navas-Martin, 2005). The S protein is also thought to interact with the M trans-membrane region (Cavanagh, 2007). The N phosphoprotein coats the viral genome for all coronaviruses and has experimentally been shown to be important in viral transcription for murine coronavirus MHV (Baric *et al.,* 1988).

Coronaviruses host and pathogenesis

Coronaviruses infect and cause respiratory, enteric and nervous system disease in a broad range of animal species (reviewed in Weiss & Navas-Martin, 2005). Evolutionary analysis of bat coronaviruses have shown that bats harbor the greatest diversity of coronaviruses and are thought to be the source of existing and emerging coronaviruses in other species (Vijaykrishna *et al*., 2007). In humans, two previously known coronaviruses include OC43 and 229E, both of which cause the common cold. Although research in coronaviruses as a group had not received much attention over the years, the emergence

of a fatal previously unknown human SARS coronavirus in 2003 drew a lot of attention and research on this group of viruses. This led to discovery of two more human coronaviruses associated with respiratory diseases in human, HKU1 and NL63 (Woo *et al*., 2005; van der Hoek *et al*., 2004, reviewed in Weiss & Navas-Martin 2005). Mouse coronaviruses are classically associated with necrotizing hepatitis, meningoencephalitis and enteritis in immunosuppressed mice and neonatal pups. Domestic mammals including porcine and bovine species are also hosts to coronaviruses that cause respiratory and enteric diseases of economic importance. In feline species a mildly pathogenic feline enteric coronavirus (FECV) causes mild, transient enteritis, while the highly virulent feline infectious peritonitis (FIPV) virus infects macrophages and is associated with disseminated granulomatous inflammation. Bird coronaviruses have in the past been known to cause respiratory disease in chickens and enteric diseases in turkeys and pheasants primarily. However new evidence suggests that other domestic and wild birds can act as hosts to known avian (Liu *et al*., 2005) and mammalian coronaviruses. Experimental inoculation of specific pathogen-free (SPF) turkey poults with DB2 calf strain of bovine coronavirus resulted in infection and clinical signs in the poults, indicating that coronaviruses may not be restricted to replicating and causing disease in single hosts (Ismael *et al*., 2001).

Coronavirus replication and transcription

Coronaviruses attach to their respective host receptor via the spike protein inducing a conformational change in the spike that result in fusion of the viral envelope and the host cell membrane. Consequently, the virus releases the nucleocapsid containing the viral genome into the cell. Like most RNA viruses, replication and transcription of

coronaviruses genomes occur in the host cell cytoplasm. Apart from replication of the RNA genome, transcription of other viral mRNAs must occur before the life cycle is complete. The coronavirus genome is the mRNA for the replicase gene and the open reading frame found on the 5' end is translated as soon as the virus enters the host cell. The translated replicase proteins then transcribe the subgenomic negative-stranded RNAs. These subgenomic RNAs, all sharing a 5' leader sequence, serve as templates for transcription of viral mRNAs that are translated into the rest of the viral proteins. The replicase also transcribes a full length negative stranded RNA which serves as a template for the genomic RNA. Both the full-length and the subgenomic negative stranded RNA molecules exist in low concentrations in the cell (reviewed in Weiss & Navas-Martin 2005; Lai, 1990).

Infectious bronchitis virus

Avian infectious bronchitis virus is the coronavirus that infects chickens. It is genetically related to the turkey coronavirus, pheasant coronavirus and other coronaviruses isolated from wild birds. These coronaviruses belong to the group 3 coronaviruses (Cavanagh & Gelb, 2008; reviewed in Armesto *et al*., 2009). IBV was the first coronavirus to be isolated and described in the 1930s in the US in samples from a respiratory disease outbreak in young chickens (Schalk & Hawn, 1931). It is a major respiratory virus of the domestic chicken and causative agent for avian infectious bronchitis in chickens. The virus primarily replicates and causes lesions in the respiratory tract with signs being more severe in young birds. The virus can also infect the reproductive tract causing reduced egg production in layers (reviewed in Cavanagh, 2003; reviewed in Collisson *et al*., 2000). Strains associated with enteric pathology (Pantin-Jackwood *et al*., 2005; Yu *et al*.,

2001) and nephropathogenicity have also been reported (Cummings, 1962; Cavanagh & Gelb, 2008; Li & Yang, 2001; Britton & Cavanagh, 2007).

IBV structure

IBV structure conforms to the general structure and composition of coronaviruses including the presence of nucleocapsid (N) protein that coats the RNA genome, spike glycoprotein (S) that is found projecting in spike-like style on the viral envelope, a small integral membrane glycoprotein (M) and also another envelope protein (E)(reviewed in Cavanagh, 2003).

The S protein of IBV can exist as both a dimer and trimer on the envelope surface. The protein is translated into a single glycoprotein that is later cleaved into amino-terminal [S1-520 amino acids (aa)] and carboxy-terminal (S2-625 aa) glycopeptides. The S2 portion remains trans-membrane and anchors the S1, the extracellular domain of the protein. Both proteins are held together by weak non-covalent forces (Cavanagh, 1983). The M protein is an important part of the virion and plays roles during virion assembly, budding and maturation. It has three trans-membrane domains incorporated in the envelope, a short extracellular amino domain and a long intracellular carboxy terminal domain. It has also been described as the most abundant coronavirus membrane protein and interacts with cellular protein actin, viral protein E, and N protein during virion assembly (Wang *et al*., 2009; Zhang *et al*., 2010). The N phosphoprotein is the most abundant structural protein in IBV and also other coronaviruses (Hiscox *et al*., 2001). It is associated with the RNA genome to form a ribonucleocapsid complex. It is also highly conserved among IBV and is found intracellularly often associated with IBV

replication complexes. It has also been detected in the nucleolar region in the nucleus (Hiscox *et al*., 2001; Chen *et al*., 2002).

The IBV genome also encodes non-structural proteins that include the RNA dependent RNA polymerase (replicase) complex that is involved in the genome replication and transcription. The gene for this protein is located in the 5' genome region as in all coronaviruses, the only protein that is translated directly from the genome. This gene has been shown to be a determinant of pathogenicity in an IBV virulent strain through reverse genetics recombination. Armesto *et al*., (2009), replaced all the genes downstream of the replicase gene of the avirulent Beaudette IBV strain with those of virulent M41 strain and found no acquired change in virulence indicating that the non-structural accessory proteins and structural proteins of the M41 strain are not the sole determinants of pathogenicity and that pathogenicity may be associated with the replicase gene in M41.The other non-structural proteins of IBV include the gene 3 and 5 found interspersed among the structural genes. Mutation of the translation start codon for gene 3 and transcription associated sequence (TAS) for gene 5 did not alter the level of replication of the IBV Beaudette strain in cultured cells suggesting they are not required for replication in cell culture (Casais *et al*., 2005; Hodgson *et al*., 2004). The lack of requirement for gene 3 in replication was also demonstrated by Shen *et al*., (2003), who studied ORF 3b sequences in mutant strains of IBV Beaudette strains recovered from virus passaged in Vero cells and embryonated chicken eggs. Youn *et al*., (2005) used molecularly cloned IBV (Beaudette strain) carrying green fluorescent gene instead of gene 5 to show that the virus replicated well in Vero cells despite the absence of this gene.

IBV genetic diversity

Existence of multiple serotypes/genotypes and variant viruses of IBV worldwide is thought to occur as a result of point mutations, insertions, deletions, and recombination (Gelb *et al*., 2005; Lee & Jackwood, 2000; Jackwood *et al*., 2003; McKinley *et al*., 2008; van Santen & Toro, 2008; Fang *et al*., 2005). Worldwide each geographical region has its own unique IBV genotypes and serotypes that seem to be endemic although some common antigenic types exist. The existence of several genotypes and serotypes necessitates frequent surveillance and characterization of prevailing genotypes to ensure effective vaccination. The different serotypes differ mainly in the sequence of amino acids in the S glycoprotein, which is the highly immunogenic surface glycoprotein that induces neutralizing antibodies in infected birds (reviewed in Cavanagh, 2007). Therefore sequencing of the S gene is used to characterize genetic diversity. Multiple serotypes may coexist in a region, which may predispose to the development of variant serotypes through recombination.

Worldwide, the Massachusetts serotype seems to be the most widely distributed and has therefore been the only approved vaccine serotype in most countries. However in many regions of the world including Europe, Africa, Middle East, Asia and Latin America, regional variants that are not fully protected by the Mass vaccine continue to be reported (reviewed in Witt *et al*., 2010). In Western Europe, S1 sequence analysis of IBV in 2419 samples collected between 2002 and 2006 showed that the Massachusetts and 793/B were the most predominant genotypes followed by Italy 02, and finally QX-like isolates. In the United Kingdom in addition to these genotypes, other genotypes detected included D274, D1466 and Arkansas (Worthington *et al*., 2009). The first isolate of QX

genotype in the United Kingdom was isolated in 2008 from a backyard chicken (Gough *et al*., 2008) but the QX genotype has recently been detected in commercial broiler flocks as well (Valastro *et al*., 2010). Many regions like Australia, New Zealand and Korea have their unique genotypes (Ignjatovic *et al*., 2006; Huang & Wang, 2006). In Australia, the strains are divided broadly into two groups, group 1 encompassing six strains that replicate both in the trachea and kidney (Vic S, N1/62, N3/62, N9/74, and N2/75) and group 2 that replicate in the trachea alone (N1/88, Q3/88 and V18/91)(Sapats *et al*., 1996). In China and Korea, circulating IBV genotypes have been characterized extensively (Liu *et al*., 2006; Lee *et al*., 2008). The Chinese strains, which are all mainly nephropathogenic, are grouped based on similarities in their S gene into 7 groups, which include strains related to Korean isolates, vaccine strains and other unique Chinese strains. Molecular typing of genotypes circulating in parts of South America (Brazil and Argentina) indicated the presence of Massachusetts genotypes. The authors suggested that the Mass circulating genotypes could be from Mass vaccine since this strain is the only one licensed for use in these regions. They also reported the presence of unique local genotypes that were found to be most prevalent (Villarreal *et al*., 2010; Rimondi *et al*., 2009). In North Africa, IBV related to the Mass and also 4/91 has been isolated together with local genotypes (Abdel-Moneim *et al*., 2006). An epidemiological survey in West Africa revealed that several European and US genotypes (793/B, Mass, D274 and B1648) are in circulation alongside other local serologically unrelated genotypes (Ducatez *et al*., 2009).

In the US, several different serotypes have been reported with some serotypes being restricted to specific geographical regions like California isolates (Jackwood *et al*.,

2007), GA98, GA08, while others like Arkansas (Ark), Connecticut (Conn), Massachusetts (Mass) and Delaware (DE072) have broader distribution. The most common however are Ark, Conn, DE072 and Mass serotypes. A survey for IBV isolates in the US for the last 11 years indicates the virus has continued to evolve and new serotypes and variants are constantly being reported (Jackwood *et al*., 2005). The Arkansas serotype is described as having higher genetic drift than the other serotypes and results in the common detection of Arkansas-like viruses (Jackwood, *2009*). There are about six serotype vaccines currently in use in the US, the most common being Mass, Conn, Ark, DE072 and GA98 (Jackwood *et al*., 2005, 2009).

IB pathogenesis and clinical signs

The first reported case of avian infectious bronchitis (IB), reported by Schalk and Hawn in 1931, manifested as a respiratory disease in baby chicks in the US and hence the name. Later, reduced egg production, reduced egg quality and nephrosis were reported in IBV-infected chickens. IBV has also been isolated from chickens with swollen head syndrome (Droual & Woolcock, 1994; Morley & Thompson, 1984) and birds with bilateral myopathy of pectoral muscles (Gough *et al*., 1992). Raj & Jones, (1996a) reported high viral loads of IBV in the ileum and diarrhea after experimental infection of day old SPF broiler chicks with IBV strain 793/B. Proventriculitis has also been observed in IBV-infected birds (Yu *et al*., 2001; Dormitorio *et al*., 2007).

The main signs seen in IBV infected chickens are attributable to respiratory tract disease. Respiratory signs observed in IBV-infected birds include gasping, rales, nasal discharge, coughing and sneezing (Cavanagh & Gelb, 2008). These signs result from replication of the IBV in the ciliated tracheal epithelium and mucus-secreting cells

(goblet cells) in the trachea of infected birds. IBV reaches peak viral load levels between 5 and 10 days post infection in uncomplicated cases (Ambali & Jones, 1990; Raj & Jones, 1997b). Initial replication of IBV occurs in the upper third of the trachea, followed by viremia, which leads to spread of the virus to other tissues (reviewed in Raj & Jones, 1997b). The virus also replicates in lung epithelial cells and air sacs where peak virus levels are found 4-11days post infection (Janse *et al*., 1994; Otsuki *et al*., 1990). Histologic lesions include mucosal thickening, serous or mucous exudates in trachea, nasal passages and sinuses. Deciliation, desquamation and infiltration of inflammatory cells (mainly heterophils, macrophages, lymphocytes and giant cells) occur in the first 2 days of infection in trachea, lungs and air sacs. Grossly, air sacs may appear cloudy and may contain deposits while the lung lesions are consistent with interstitial pneumonia (King & Cavanagh, 1991). In studies using tracheal organ cultures, ciliary stasis is always seen, a characteristic used experimentally as measure of virulence and an assay for IBV (Raj & Jones, 1997b).

IBV also infects and causes pathology in the reproductive tract both in young chicks and laying hens (Benyeda *et al*., 2010). In laying hens some strains of IBV result in decreased egg production, poor shell quality (Jolly *et al*., 2005) and poor egg internal quality (reviewed in Raj & Jones, 1997b). These effects are a result of infection of the oviduct epithelium and glandular cells resulting in glandular hypoplasia. Glandular hypoplasia results in decrease of synthesis of the albumen proteins, especially those that make up the thick albumen, leading to 'watery whites.' Loss of oviduct cilia, lymphocytic infiltration in lamina propria, glandular dilation and accumulation of serum-like material in oviduct are common changes in layers infected with some IBV strains (Benyeda *et al*.,

2010). Australian strains were found to be only mildly pathogenic in the reproductive tract but caused loss of shell color (Choulsakar & Roberts, 2007; Choulsakar *et al*., 2009). In young female chicks of less than 2 weeks of age, infection of the oviduct may lead to permanent damage to the oviduct and also chickens that lay abnormally when they reach reproductive age. Arrested development of the reproductive tract, occlusion of the oviduct and formation of cysts has also been reported in infected young chicks (Crinion *et al*., 1971). The severity of these signs and concurrent presence of respiratory signs depend on the strain of the virus and also the period of lay when the chicken is infected. The M41 strain of IBV is thought to be the most virulent for both mature and immature female oviducts (reviewed in Raj & Jones, 1997b). In males, epididymal lithiasis and reduced fertility in live and killed vaccine-vaccinated roosters (Boltz *et al*., 2006; Jackson *et al*., 2006) and naturally-infected roosters (Villarreal *et al*., 2007; Boltz *et al*., 2004) has been reported. Some authors have reported detection of IBV without lesions in the testicular tissues in experimentally-infected roosters (Amjad *et al*., 2009; Gallardo *et al*., 2011).

The first nephropathogenic IBV was reported in Australian chickens in 1962 (T strain) (Cumming, 1962). Later, nephropathogenic strains were reported in the US and Europe, and currently these types are reported worldwide including Asia and Africa (Butcher *et al.,* 1990; Zieglar *et al*., 2002; Choi *et al*., 2009; Liu & Kong, 2004; Abdel-Moneim *et al*., 2006; Bayry *et al*., 2005; Bouqdaoui *et al*., 2005). In embryonating eggs where IBV vaccine viruses are cultivated, kidney lesions (interstitial nephritis accompanied by urate formation) are a common finding suggesting that most strains of IBV can infect and replicate in kidney tissues. The virus replicates in the renal tubules

and ducts resulting in electrolyte and fluid imbalance and finally renal failure (Raj & Jones, 1997b; Cavanagh, 2007). Although different strains cause similar lesions in the kidney, the severity of these lesions depends on the strain and often the viral titer does not correlate with the severity of the kidney lesions (Ambali & Jones, 1991).

Although many strains of IBV are shed through the fecal route in both vaccinated and naturally infected birds and can be recovered from cloacal swabs, there has been no IBV strain described as severely enteropathogenic. It is well known though, that IBV replicates in the gut, resulting in fecal shedding of the virus. The shedding of virus through this route is known to play a role in transmission (Cavanagh, 2007). Although IBV has been detected in esophageal swabs, it is not clear if the virus comes directly from viral replication in the esophagus or whether it is virus originating from trachea that is expelled (Raj, 1997). Proventriculitis was experimentally reproduced in both one-day-old SPF and IBDV/IBV antibody positive commercial broilers by oral gavage of proventricular homogenates from broilers with naturally occurring proventriculitis. All the birds developed proventriculitis and IBV was one of the viruses detected in these birds (Pantin-Jackwood *et al*., 2005). Replication of IBV in the proventricular tissues is thought to occur in the epithelial cells and the proventriculus is ranked third as a replication site after trachea and oviduct tissues in vitro (Raj & Jones, 1997b). In the lower gut tissues, IBV has been isolated from the cecal tissues (Lucio & Fabricant, 1990; Butcher & Winterfield, 1990; Escorcia *et al*., 2000; Alvarado *et al*., 2006) infecting cells that resemble macrophages and lymphoid cells (Owen *et al*., 1991). Ambali & Jones (1990) demonstrated the presence of IBV antigen in enterocytes at the tips of villi in

ileum and rectum. Some strains of IBV are described as more enterotropic by the fact that they are isolated from the gut for a longer period (Raj & Jones, 1996a).

IBV has been isolated from Harderian gland tissues and IBV antigen detected in stroma of infected gland by immunofluorescent staining. Eye drop vaccination resulted in initial desquamation of tubular epithelium of Harderian gland, damage to plasma cells and recovery within 14 days (reviewed in Raj & Jones, 1997b; Toro *et al*., 1996).

Severity of IBV disease and involvement of different tissues is under influence of many factors. The strain of IBV involved is a major determinant as different strains induce varying degrees of pathology in affected tissues (Ignjatovic *et al*., 2002; Albassam *et al*., 1986). The age of the chickens seems to play a role as the disease is more severe and tends to persist longer in young chickens than older chickens in experimentally infected birds (Cavanagh & Gelb, 2008). Less pathology in kidney and resistance to kidney and oviduct infection is also shown to increase with age. Breed of chicken, nutrition, temperature, immunosuppression and the presence of concurrent bacterial infections have all been documented to alter IBV disease severity (Raj & Jones, 1997b).

Immunity to IBV

Immunity to IBV is from both humoral and cell mediated immunity after natural infection or vaccination. Passive transfer of maternally derived antibodies also occurs in chicks hatched from immune parents (Mondal & Naqi, 2001). Chickens recovering from IBV infection are usually immune to the infecting strain but may not be immune to other heterologous strains (Cavanagh & Gelb, 2008) meaning that immunity is usually serotype specific. Most studies have focused on humoral response analyzing antibodies in serum,

lachrymal fluid, and trachea (Gomez & Raggi, 1974; MacDonald *et al*., 1981; Cook *et al*., 1986; Da Silva Martins *et al*., 1991; Toro *et al*., 1994; Gelb *et al*., 1998).

Experimentally, protective immunity to IBV is analyzed by recovery of virus from challenged birds, analysis of respiratory signs, and also histological evaluation of tracheal lesions (Winterfield *et al*., 1972; Yachida *et al*., 1985). Analysis of monoclonal antibodies against IBV generated in murine hybridoma cells has shown that neutralizing and hemagglutinating antibodies are against the IBV S1 protein, therefore this membrane protein has been considered as most important in induction of protective antibodies. Epitopes for cross-reactive antibodies were detected in the S2 fragment of the S protein (Mockett *et al*., 1984) and also the nucleoprotein (N) protein (Seah *et al*., 2000; Ignjatovic & McWaters, 1991) although their role in protection against infection is not clear. Antibodies against IBV including IgM, IgA and IgG and have been detected in serum, trachea, lachrymal fluid and oviduct (Raj & Jones, 1996) while IBV-specific IgA and IgM secreting cells have been observed by enzyme-linked immuno-spot (ELISPOT) assays in the Harderian gland and cecal tissues from chickens inoculated with IBV (van Ginkel *et al*., 2008). Most tests for seroconversion post vaccination or infection use detection of antibodies in sera. The major antibodies in sera are IgG which are detected as early as four days post inoculation and peak at 21 days, consequently remaining high in titer for a long time (Raj & Jones, 1997). IBV-specific IgM is only transiently detected, peaking at 8 days and declining to pre-vaccination levels by 12 days post vaccination. IgM antibodies have been shown to be useful in diagnosis of recent infections (Da Silva Martins *et al*., 1991). IBV-specific IgA has been detected in lachrymal fluid after experimental vaccination (Raj & Jones, 1996; Toro *et al*., 1994; Cook *et al*., 1992) and in

higher amounts than IgG (Toro *et al*., 1994). The Harderian gland has been shown to be rich in plasma cells that are the source of these antibodies. Most studies have found no correlation between amount of serum antibodies, antibodies in tears and the level of protection (Cavanagh, 2007; Cavanagh & Gelb, 2008; Gelb *et al*., 1998; Mondal & Naqi, 2001; Yachida *et al*., 1985). Some authors however found association of lachrymal IBV-specific IgA and protection against IBV (Toro & Fernandez, 1994, Cook *et al*., 1992) and suggested IBV-specific IgA in tears as good measure of protection.

The role of cell-mediated immunity in protection against IBV is documented (Janse *et al*., 1994; Boots *et al*., 1992; Fulton *et al*., 1993; Seo & Collisson, 1997; Collisson *et al*., 2000; Pei *et al*., 2003; Raj & Jones, 1997a). Cytotoxic T lymphocyte (CTL) activity was shown to peak 10 days post infection, was MHC restricted and correlated well with initial viral clearance. Adoptive transfer of IBV-primed CD8+ alpha-beta T cells protected chicks from acute infection (Seo *et al*., 2000; Collisson *et al*., 2000). The amount of IBV in the lungs and the kidney correlated with the level of CTL activity. CTL epitopes have been mapped to the carboxyl end of the N protein and middle region of the S1 glycoprotein (Boots *et al*., 1991; Raj & Jones, 1997a; Seo *et al*., 1997; Collisson *et al*., 2000).

Genetic resistance to IBV is not fully investigated. Resistance is seen in terms of time to recovery and severity of the lesions induced after IBV infection. Otsuki *et al*., (1990) reported equal susceptibility to IBV infection in chickens of different inbred lines (Line C vs. line 151). However, recovery was quicker and viral load much lower in line C than line 151 chickens. The authors suggested that these differences in resistance may be as a result of differential immune response in the different lines of chickens. A

retrospective study in young chickens accidentally vaccinated with a moderately attenuated strain of IBV showed that resistance to IBV is associated with the MHC (*B*) haplotypes carried by the chicken (Bacon *et al*., 2004). These chickens were all susceptible to infection but those with *B**15 haplotype recovered faster, had less severe and transient tracheal lesions, and recorded lower mortality than those with *B**13 or *B**21 haplotypes. Similarly, Joiner *et al*., (2007) found significantly higher incidence of respiratory signs in *B**21 than *B**15 haplotype -carrying chickens vaccinated and challenged with an Ark serotype IBV. However, no differences in the duration and severity of respiratory signs and histopathology lesions were found between *B**15 and *B**21 haplotype chickens in this study.

Diagnosis and detection of IBV

Diagnosis of IBV infection has historically been based on the presence of clinical signs, presence of lesions at postmortem, seroconversion, virus isolation and IBV antigen detection. More recently nucleic acid (RNA) analysis has become a common method of detection (Cavanagh & Gelb, 2008). Serologically, IBV infections are diagnosed using hemagglutination inhibition (HI), agar gel precipitation (AGPT) and enzyme linked immunosorbent assay (ELISA) (reviewed in de Wit, 2000).

Because the primary organ of infection by IBV is the trachea, tracheal swabs/tissues are the primary samples where IBV is analyzed. Cloacal swabs and cecal tonsils may also be of value in viral isolation especially at post mortem and where infection has been present for over a week (Cavanagh & Gelb, 2008; de Wit, 2000). Isolation of IBV in culture (embryos, tracheal organ cultures and cell cultures), detection of IBV antigen using immunofluorescence or immunoperoxidase staining, and nucleic

acid analysis are the main ways of confirming the presence of IBV. Usually none of these methods is in itself sufficient and often a combination of these methods is applied. Virus isolation using embryonated eggs is widely applied and maximum virus titer in allantoic fluid is reached within 1 to 2 days for egg-adapted strains. However for field isolates or non-egg-adapted strains, several blind passages are recommended before samples can be declared true negatives (Cavanagh & Gelb, 2008). This process is time consuming if used alone and it is therefore often combined with IBV antigen detection after a few passages to reduce waiting time. IBV isolation using tracheal organ cultures (TOCs) from 20 day old embryos is successful in isolation, titration and serotyping of IBV and usually no adaptation is required (de Wit, 2000). Ciliostasis is usually induced 3 to 4 days post infection although it differs between strains. Since other agents can also induce ciliostasis, confirmation of field isolates requires other IBV-specific tests.

Detection of IBV antigen is another method commonly applied for IBV detection. IBV antigen detection assays use IBV-specific antibodies either as polyclonal or monoclonal antibodies. The assays include agar gel immunoprecipitation, immunofluorescence (IFA) and immunoperoxidase assay (IPA) (reviewed in de Wit, 2000). Use of monoclonal antibodies prevents non-specific reactions (Yagyu & Ohta, 1990). An antigen-capture ELISA for detection of IBV has been developed that uses capture monoclonal- or polyclonal-antibodies coated onto plates. These antibodies bind IBV antigen in test samples and then labeled secondary antibodies act as detecting antibodies. This assay was found to successfully detect IBV antigen in tissues from experimentally and field infected birds. High correlation between this assay and viral isolation was reported (Ignjatovic & Ashton, 1996).

The use of nucleic acid assays for IBV detection has been widely applied since Brown *et al.,* 1984 reported use of radiolabelled cDNA probe complementary to IBV RNA (from Beaudette strain UTR) for confirmation of IBV in virus isolates. Later, other DNA hybridization probes to detect IBV were developed (Callison *et al*., 1990; Jackwood *et al*., 1992; Kwon *et al*., 1993). Amplification of the N gene followed by sequencing for IBV strain differentiation was reported by Zwaagstra *et al*. (1992), while Falcone *et al*. (1997) reported development of a nested RT-PCR that targeted the N gene for rapid diagnosis and strain differentiation in allantoic fluid, cell cultures and infected tissues. Furthermore, universal oligonucleotide primers that are able to detect many different strains of IBV have been published targeting either the N gene, UTR region, the S2 gene (Handberg *et al*., 1999; Adzhar *et al*., 1996) or S1 (Keeler *et al*., 1998; Handberg *et al*., 1999). A multiplex reverse transcriptase PCR (RT-PCR) reaction that can differentiate different IBV strains has also been reported (Liu *et al*., 2003). RFLP of RT-PCR amplified products was also able to differentiate IBV strains accurately (Kwon *et al*., 1993; Mardani *et al*., 2006). Techniques with high sensitivity and ability to quantify IBV in samples have also been developed and are now widely applied (Callison *et al*., 2006). A highly specific and fast assay that provides rapid detection and non-subjective characterization of IBV using high resolution melting curve analysis has also been developed (Hewson *et al*., 2009).

IBV evolution

The fact that IBV is constantly evolving is evidenced by the existence of several serotypes and the frequent detection of numerous variants worldwide. IBV evolution mechanisms have been described as complex, unpredictable and poorly understood

(Sapats *et al*., 1996; Jia & Naqi, 1997; Ignjatovic *et al*., 2006). IBV evolution is under influence of factors that are inherent in the virus, host mediated and also environmental. Recombination and mutations (deletions, insertions and point mutations) are thought to be the major mechanisms that drive evolution in IBV (Cavanagh *et al*., 1992; Jia *et al*., 1995; Kusters *et al*., 1990; Mardani *et al*., 2010).

Inherent viral use of RNA polymerase for replication results in the introduction of deletions, insertions and point mutations within the IBV genome during replication. This phenomenon is responsible for the existence of RNA viruses as quasispecies (Domingo *et al*., 1985). For IBV, mutations occurring within the S, N and the 3' UTR genes are associated with the emergence of new variants and strains (Ignjatovic *et al.,* 2006; Lee & Jackwood, 2001) and sometimes serotypes through antigenic drift (Jackwood *et al*., 2005).

Continued use of live attenuated vaccines leads to positive selection of IBV vaccine viruses and variant viruses by the vaccinated chicken's immune pressure possibly leading to emergence of more virulent viruses (Lee & Jackwood, 2001a; Dolz *et al.*, 2008). Use of live attenuated vaccines often of multiple serotypes may also lead to circulation and sometimes persistence of IBV vaccine viruses in the field. Persisting vaccine viruses may continue to mutate and also offer genetic material for recombination leading to generation of new IBV viruses (van Santen & Toro, 2008). Evidence of recombination among IBV viruses has been documented. Co-infection of embryonating eggs with M41 and Beaudette strain of IBV resulted in isolation of infectious hybrid genome of the two strains (Kottier *et al*., 1995). The virulent DE072 isolate is also thought to be derived from recombination of a Netherlands vaccine strain (D1466) and

the CU-T2 based on high homology of the S1 and S2 genes with D1466 and homology in other regions with the CU-T2 (Lee & Jackwood, 2000). Non-synonymous mutations within the hypervariable S1 region of DE072 combined with positive immune selection are thought to have given rise to the genetically related but antigenically different Georgia 98 (GA98) serotype (Lee & Jackwood, 2001). The contribution of multiple live attenuated vaccines in generation of new IBV variants has been suggested. Jia *et al*., 1995 described a variant of IBV, CU-T2 that was found to contain virus-neutralizing and serotype-specific epitopes within its S1 glycoprotein belonging to both Ark and Mass serotypes. The S2 glycoprotein sequence also arose from independent recombination event. Further genetic analysis of the variant genome revealed that half of its nucleoprotein gene was replaced by a sequence from H52 vaccine strain indicating a three-strain recombination in this variant. Similarly Wang *et al*., (1993) reported isolation of a field strain containing genome sections of both M41 and Ark 99 that showed recombination within the S1 gene region. Recently by sequencing and phylogenetic analysis of the a virulent ArkDPI strain, it was suggested that this strain may have originated from recombination event involving Conn, Ark 99 and JMK strains since regions within the ArkDPI strain shared high sequence homology with each of these strains (Ammayappan *et al*., 2008). The Cal99 strain is also thought to have arisen by recombination events between the vaccine strains in use in California. The S1, S2 and M gene sequences share homology with the Ark, Conn and Mass IBV strains respectively suggesting that this strain may have arisen from these three strains (Mondal & Cardona, 2007).

Control of IBV

Ideally, good biosecurity, good management practices that include "all in all out" practice, cleaning and disinfection, strict isolation and repopulation with day old chicks only, are helpful in controlling IBV infection (Cavanagh & Gelb, 2008). However, current broiler commercial production systems have limited clean out time and layer systems often have multiple age groups, which complicates control of this disease through management practices. Airborne transmission of the virus also renders biosecurity measures ineffective. In these circumstances, immunization remains the only viable option for control of IBV (Cavanagh & Gelb, 2008; Mardani *et al*., 2006a).

Vaccination against IBV is through use of both live attenuated and inactivated vaccines. Live vaccines are administered to broiler type birds while in layers and breeders, live vaccines are given to pullets before lay as a primer and inactivated vaccines are given later just before lay to boost immunity. In some cases, vaccination during lay is recommended in single age group laying flocks to reduce production losses in susceptible flocks. In the US layers are vaccinated at 8-10 week intervals during lay with the Mass serotype via drinking water. Broilers are vaccinated with live IBV vaccine at the hatchery, followed by a live boost at 18-21 days of age in the field (Cavanagh & Gelb, 2008).

Worldwide, the Massachusetts serotype vaccine is the most common strain that is licensed for use as vaccine since it is believed that this serotype is the most widespread in distribution (de Witt *et al*., 2010; Cavanagh & Gelb, 2008). In Europe, the Mass serotype vaccines are found in different forms including H120, Ma5 and Modified Mass (MM), which are given in monovalent and occasionally in bivalent forms although the bivalent

forms are not allowed in some European countries (Jones, 2010). In addition, vaccines of other IBV serotypes specific to Europe [D274, D1466, and 4/91 (also referred to as CR88 and 793/B)] and Asia (QX) are also used (Cavanagh & Gelb, 2008) usually after priming with the Mass serotypes (Jones, 2010). There are reports that vaccination with two heterologous strains of IBV usually two weeks apart results in broader protection against IBV serotypes than when the vaccines are administered simultaneously (Cook *et al*., 1999; Terregino *et al*., 2008). In Australia and New Zealand, only IBV vaccines developed from local strains are used (Ignjatovic *et al*., 2006; McFarlane, 2008, Cavanagh & Gelb, 2008). In the US, six different serotypes are routinely vaccinated against with the Massachusetts, Arkansas and the Connecticut serotypes being the most commonly used. Other vaccines used in the US include GA98, GA08 and the DE072 serotypes, all of which are used regionally (Jackwood *et al*., 2005). Since in many regions, several serotypes are in circulation at any time, combined serotype vaccines are given simultaneously. Booster vaccination is done for all commercial chickens and the numbers of boosters depend on the breed, with broilers usually being vaccinated at hatch and again two to three weeks later while in layers and breeders additional vaccinations are required for full protection (Jackwood *et al*., 2009).

Ark IBV serotype vaccine use and concerns in the US

In the United States, despite the use of the Ark serotype vaccines alongside other serotype vaccines, cases of ArkDPI type IBV isolations continue to be reported in increasing frequencies in the southeastern U.S. (Nix *et al*., 2000; Toro *et al*., 2006; Jackwood *et al*., 2005). Molecular typing of IBV field isolates collected over an eleven-year period in the US revealed that ArkDPI viruses were the most prevalent, ranging

from 25-65% each year (Jackwood *et al*., 2005). Similarly analysis of avian cases with respiratory signs submitted to Alabama state diagnostic laboratories over a five year period indicated that Ark-type IBV isolates were the most common (Toro *et al*., 2006). Ark vaccine viruses were the predominant isolates in samples taken several days after vaccinations with multiple IBV serotype combined vaccines indicating that Ark vaccine viruses persist in vaccinated flocks longer than other serotype vaccines (Jackwood *et al*., 2009). In addition, the Ark vaccines were also found to offer less protection (37.5-65%) as compared to the DE072 serotype (100%) after administration of these vaccines together in the field (Jackwood *et al*., 2009). Previous experimental studies comparing isolation of Ark and Mass vaccine viruses reported that Mass vaccine viruses are detected earlier while Ark vaccines were detected later on (Alvarado *et al*., 2006). Furthermore Jackwood *et al*., (2005) also reported new Ark-like variants being diagnosed each year for the eleven years indicating a potential for genetic drift phenomenon within Ark-serotype IBV.

ArkDPI vaccine history and characteristics

The ArkDPI vaccine is widely used in the US and also in Europe. The ArkDPI vaccine strain was derived from an Ark-serotype isolate obtained from broiler chickens in the Delmarva Peninsula. This isolate was passaged 50 times in embryonated eggs, tested and found to be of reduced virulence in chickens (Gelb & Cloud, 1983). In comparison with the unattenuated virus, the 50^{th} passage virus caused less severe respiratory signs and the appearance of these signs was delayed. The attenuated stock was distributed to vaccine companies who attenuated it further by more passages (reviewed in van Santen and Toro,

2008). Currently, at least four different commercial ArkDPI-derived vaccines are widely used in the United States (van Santen and Toro, 2008).

Molecular analysis using RFLP and sequencing of a section of the S1 gene of two commercial ArkDPI vaccines, original stock distributed to vaccine companies and the original unattenuated isolate revealed the presence of a minor viral subpopulation similar to a pathogenic Ark subtype detected in the Delmarva Peninsula (Nix *et al*., 2000). Although the authors suggested that the ArkDPI vaccines could have been the source of the pathogenic Ark subtype detected, they consider vaccine heterogeneity desirable to increase the breadth of protection (Nix *et al*., 2000). van Santen & Toro, (2008) examined the S1 gene of the four ArkDPI-derived vaccines currently in use in the US before and after a single passage in chickens. They found different degrees of heterogeneity among these vaccines, which ranged from no apparent heterogeneity to heterogeneity in up to 20 nucleotide positions in the S gene. Most of these nucleotide differences were non-synonymous. Similarly, McKinley *et al*., (2008) found differences in the S1 gene of three different ArkDPI derived vaccines from different manufacturers. They also found single amino acid differences between different lots of vaccines from the same manufacturer. Analysis of viruses recovered during a single passage of these ArkDPI vaccines in chickens, found that minor viral subpopulations within these vaccines were selected as early as three days post vaccination in inoculated chickens (van Santen & Toro, 2008; McKinley *et al*., 2008; Gallardo *et al*., 2010). The selected viral subpopulation S1 genes differed from the major vaccine subpopulation consensus at 5 to 11 codons. The proportions of these selected viral subpopulations in the vaccine vials differ amongst the ArkDPI vaccines. These differences may affect the induction of immune response and/or

induction of pathology in the respiratory system (van Santen & Toro, 2008). The presence of these viral subpopulations within these vaccines may also favor viral survival through selection leading to persistence. Persisting vaccine viruses may offer genetic material for mutation and recombination leading to emergence of virulent viruses (Domingo *et al*., 1998; van Santen & Toro, 2008; McKinley *et al*., 2008).

IBV vaccine administration in chickens

Administration of IBV vaccine as in other poultry vaccines in commercial systems often involves thousands of birds and mass administration is the only practical way of administering these vaccines. Mass application methods include coarse spray, aerosol and drinking water application (reviewed in Cavanagh & Gelb 2008). Mass administration methods are popular due to their convenience when dealing with large flock sizes. However these methods have shortcomings ranging from severe vaccine reaction, non-uniform flock coverage, vaccine dose variability, and vaccine inactivation in water (Jackwood *et al*., 2009; Cavanagh & Gelb 2008; de Witt *et al*., 2010; Fulton *et al*., 2000).

Vaccine dose has been shown to affect induction of humoral immune response in experimentally vaccinated chickens. In an experiment comparing different IBV vaccine doses, Toro *et al*. (1997) reported no detectable systemic IBV-specific antibodies in chickens vaccinated with IBV dose of 10^4 50% embryo infectious doses (EID_{50}), and no local (IgA) or systemic antibodies in chickens receiving the lowest vaccine dose (10^2 EID_{50}). Higher doses (10^6 EID_{50}) however, induced both systemic and local antibodies irrespective of the route of administration.

Non-uniform flock coverage in large chicken flocks results in the presence of non-immune contact birds that may be infected by viruses shed by birds that received the initial vaccine virus. While this mode of virus transfer in vaccinated flocks may help to induce immunity in this group of contact birds (Matthjis *et al*., 2008), a major concern is the vaccine viruses 'rolling reaction' that has been reported to result in increased vaccine virus virulence (Hopkins & Yoder, 1986; McKinley *et al*., 2008).

In addition IBV vaccines are often given as multiple serotype vaccines or in combination with other vaccines like Newcastle disease vaccine (Wakenell & Sharma, 1986). Multiple vaccine administration may provide protection against several agents simultaneously, saving time and costs of several vaccinations. This is especially important where multiple serotypes of a disease agent exist in a region and repeated vaccinations are required for these agents. It is also helpful when large numbers of subjects are involved, as in the poultry industry. However, concerns exist in these circumstances over the issue of vaccine interference. Certain vaccines that are protective when given singly have been associated with poor immune induction when given together with other vaccines (Vidor, 2007; Gagic *et al*., 1999). Interference of immune induction between IBV serotypes was reported as early as 1968 where the more virulent Mass-41 strain interfered with the milder Connecticut strain neutralizing antibody induction but not vice versa when the vaccines were given simultaneously (Winterfield, 1968).

Research Objectives

Factors responsible for high frequency of isolation of ArkDPI genotype IBV, in the poultry production belt in the southeastern US remain to be elucidated. The presence of

viral subpopulations within the ArkDPI derived vaccines that more efficiently replicate in chickens coupled with the practice of multiple serotype vaccine administration in the field might play a role. The specific objectives for this work are:

1. Determine the significance of different proportions of selectable viral subpopulations within ArkDPI vaccines

2. Compare humoral immune response and persistence of vaccine viruses in chickens vaccinated with two different Ark serotype vaccines

3. Determine transmission ability of various Ark vaccine viral subpopulations to non-vaccinated contact birds.

4. Evaluate the replication and selection characteristics of Ark vaccine viruses when given in combination with Mass serotype vaccine.

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CHAPTER 2

SIGNIFICANCE OF DIFFERENCES IN PROPORTIONS OF SPECIFIC MINOR VIRAL SUBPOPULATIONS WITHIN ARK-TYPE INFECTIOUS BRONCHITIS VACCINES

Abstract

In the United States, Ark-like IBV viruses continue to be the most frequent isolates in respiratory cases confirmed to be due to infectious bronchitis. The reason behind the predominance of Ark-like viruses is unclear. Ark serotype vaccines in use in the US today have been found to contain multiple viral subpopulations, a small fraction of which is selected as early as three days post vaccination. These selected subpopulations exist in the different Ark serotype vaccines, which are all ArkDPI-derived, in differing proportions. This study investigated the significance of differing proportions of selected subpopulations in ArkDPI vaccines on vaccination outcome. In general, Ark vaccines with higher proportions of the selected subpopulations induced significantly higher viral loads, incidence of respiratory signs, and more severe tracheal lesions but also a higher immune response. One Ark vaccine despite having a low viral load also induced a high incidence of respiratory signs and tracheal lesions. This vaccine also resulted in heterogeneous subpopulations being established in vaccinated chickens. Sometimes individual chickens had more than one subpopulation detected in them. The presence of viral subpopulations inducing severe tracheal lesions in vaccinated chickens may suggest existence of residual virulent subpopulations within the Ark vaccines.

Although in some cases the same Ark vaccine inducing severe lesions was also associated with a stronger immune response, one of the Ark vaccines induced severe lesions but did not enhance immunity. Analysis of these Ark vaccine viral subpopulations, preferably in their cloned state, might reveal their benefit as vaccine candidates, if any, or contributions to undesirable vaccine outcomes.

Introduction

Infectious bronchitis virus (IBV) causes an economically important respiratory disease in chickens worldwide. It is highly infectious, affecting and causing great economic losses in birds of all ages (Cavanagh, 2007). Losses are due to high mortality in young birds, reduced weight gain accompanied by post slaughter carcass condemnation in surviving meat birds, reduced egg production in layers and condemnation of eggs due to deformation and poor shell quality **(**Cavanagh, 2005; Britton & Cavanagh, 2007**)**. Although vaccination has reduced the losses by tremendous magnitudes, the disease continues to be ranked among the most important diseases affecting chickens in the United States (US) and Europe (Jackwood, 2007; Bijlenga *et al*., 2004; Cavanagh & Naqi, 2003; Cavanagh, 2007). The prevalence of this disease is partly due to numerous viral serotypes, poor cross protection between serotypes and the rapid and unpredictable appearance of new serotypes through spontaneous mutations or recombination (Cavanagh *et al*., 1992; Jia *et al*., 1995; Cavanagh, 2005; Bijlenga *et al*., 2004).

IBV is found in group 3 of coronaviruses, which also includes the turkey coronavirus (Guy, 2000), pheasant (Cavanagh *et al*., 2002) and other coronaviruses reported in wild birds (Woo *et al*., 2006; Jonnassen *et al*., 2005). As the name suggests,

IBV has widely been known to cause respiratory disease although it is also known to infect other epithelial and mucus producing cells of non-respiratory tissues.

The IBV genome encodes four major structural proteins that include major envelope glycoprotein (spike, S), membrane glycoprotein (M), nucleocapsid (N) and small envelope protein (E). The S protein (spike) is made up of two subunits, S1 and S2. S1 projects from the virion envelope and hence its name, spike protein, while the S2 anchors the protein onto the membrane. The S1 subunit (S1) of the S protein induces neutralizing antibodies to IBV (Cavanagh *et al*., 1984,1988; Kant *et al*., 1992; Ignajatovic *et al*., 1994; Mockett *et al*., 1984). In addition, changes in the S1 protein are associated with adaptation to different hosts and tissues for IBV and other coronaviruses (Gallagher & Buchmeier, 2001;Kuo *et al*., 2000;Casais *et al*., 2003; Fang *et al*., 2005; Ammayappan *et al*., 2009; Cavanagh *et al*., 2005). Although differences in the S1 protein determine serotypes and tissue tropisms almost exclusively, changes in other genes within the virus are thought to be important in generation of highly attenuated phenotypes within IBV (Hodgson *et al*., 2004; Armesto *et al*., 2009).

For many years control of IBV worldwide has been mainly through the use of live attenuated vaccines. Several serotypes of IBV exist worldwide and hence several vaccine serotypes are available (reviewed in Britton & Cavanagh, 2007). IBV vaccine attenuation involves multiple passages (usually more than 75) in embryonating eggs, resulting in reduced virulence in chickens and increased adaptation to the chicken embryo (Gelb & Cloud, 1983; Bijlenga *et al*., 2004; Huang & Wang, 2006; Jackwood *et al*., 2003). However the balance between sufficient replication to induce immunity and ability to cause pathology is a subject of concern in the development and use of live attenuated

vaccines (Bijlenga *et al*., 2004). While live attenuated vaccines are routinely checked for reduced virulence before release for use in animals, concern still exists regarding the stability of the vaccine viruses, in particular reversion to virulence when administered to commercially reared flocks. Back-passage of live attenuated IBV vaccines in chickens has been shown to result in generation of more fit variants that produce more severe pathology and predispose chickens to secondary bacterial infections (Hopkins & Yoder 1984; Hopkins & Yoder, 1986). Therefore circulation of IBV vaccine virus among chickens in vaccinated flocks due to uneven vaccination may result in generation of viruses that persist and cause disease in commercial flocks after vaccination.

In the US, the most commonly used IBV vaccine serotypes are Massachusetts, Arkansas, and Connecticut. The serotypes GA98 and DE072 are also used regionally (Jackwood *et al*., 2005). The Arkansas Delmarva Poultry Industries (ArkDPI) strain was first isolated in the Delmarva Peninsula from broiler chickens after which it was passaged 50 times in embryonating eggs until virulence was reduced (Gelb & Cloud, 1983). This attenuated stock was distributed to vaccine companies who passaged it further in embryonating eggs to generate the commercial ArkDPI-derived Ark serotype IBV vaccines currently used. The ArkDPI-derived IBV vaccine was first licensed in 1985.

Despite the use of the ArkDPI-derived vaccines, cases of Ark-type IBV isolations continue to be reported in increasing frequencies in regions where the vaccine is being used. Molecular typing of IBV field isolates collected from the southeastern US over an eleven year period indicated that the Ark-DPI-like viruses were the most frequently isolated ranging from 25-65% each year (Jackwood *et al*., 2005). In addition, Toro *et al*., 2006 also reported that Ark-type IBV isolates were the most common in avian cases

submitted to the Alabama State Veterinary Diagnostic Laboratories with respiratory signs over a five-year period. Furthermore, persistence of Ark IBV vaccinal strains in vaccinated flocks for a long time has recently been documented (Jackwood *et al*., 2009). In the latter study, monitoring of chickens vaccinated by various routes/methods with a combination of the various serotypes of IBV circulating in the region revealed that persisting vaccine viruses were Ark. This study also reported detection of field infections with Ark-like isolates in non-vaccinated flocks in an area where the Ark vaccines were being used as live vaccine. Jackwood *et al.,* (2005) also reported new Ark-like variants being diagnosed each year for the eleven-year period examined, indicating a potential for genetic drift among Ark-like IBV.

Recent studies on the characteristics of Ark serotype vaccines (ArkDPI-derived) currently in use in the US documented that they are heterogeneous in nature and that some minor viral subpopulations found in these vaccines apparently replicate better and are selected in vaccinated birds by three days post-vaccination (van Santen & Toro, 2008; McKinley *et al*., 2008). These Ark vaccines were found to contain these minor selectable subpopulations in differing proportions. Two of the Ark vaccines coded A and C contained a higher proportion of the selected subpopulations than two other vaccines coded B and D. In vaccines B and D, the selected subpopulations were detectable only by PCR with primers specific for selected subpopulations (van Santen & Toro, 2008). McKinley *et al*. (2008) also reported mutations of vaccine viruses occurring during replication in vaccinated chickens.

It is not known if the presence of differing proportions of viral subpopulations able to replicate better in chickens, as documented in Ark-DPI-derived vaccines, play any

role in determining the outcome of vaccination in vaccinated chickens. In this study we hypothesized that ArkDPI-derived IBV vaccines containing higher proportions of the subpopulation(s) able to efficiently replicate in the upper respiratory tract of chickens would produce higher viral loads, more severe "vaccine reaction" (respiratory signs, tracheal damage) and more rapid/vigorous immune response compared to vaccines with very little selectable subpopulation. The results of the study contribute information useful for designing safer and more effective IBV vaccines.

Materials and Methods

Vaccines

Four single entity commercial live-attenuated IBV Ark-serotype vaccines originally derived from the ArkDPI strain were used in this study. The vaccines were coded, A, B, C, and D, corresponding to the same codes used in a previous study, van Santen & Toro (2008). Lyophilized vaccines were reconstituted in tryptose phosphate broth containing antibiotics and antimycotics. The viruses were titered in 10-day-old chicken embryos. Titers were calculated by the method of Reed and Muench (Villegas *et al*., 2008).

Experimental design

Chickens were hatched from specific pathogen free eggs obtained from Sunrise Farms (Catskill, New York). Thirty-six chickens in each of four groups were individually vaccinated with IBV Ark vaccine A, B, C or D one day after hatch. Each bird received 4 X 10^4 embryo infectious dose (EID_{50}) divided equally in both eyes and nares. Nineteen control chickens were also included as unvaccinated controls. Each group of birds receiving the same vaccine was housed in Horsfall-type isolators. Absolute care was

taken between sampling from groups administered different vaccines to ensure the groups were not cross-contaminated.

Tears from ten individual chickens per vaccine group were collected 3, 5, 8, 11, and 15 DPV as previously described (Toro *et al*., 1993) and immediately cooled to 4°C in ice until taken to the laboratory where they were stored at -20°C. Tears were used for RNA extraction. RNA extracted from tears was used for viral load quantification using quantitative RT-PCR and also cDNA generation for sequencing.

Vaccinated birds were also analyzed for the extent of vaccine reaction physically by monitoring the presence of respiratory signs and histologically by examining tracheal lesions. Individual birds were physically monitored for the presence of audible respiratory sounds (rales) daily between 4-7 days post vaccination and every 2 or 3 days between 9 and 14 days post vaccination by bringing the head of each bird close to the examiner's ear. The number of positive birds in each group was recorded for each sampling day.

Six chickens in each group were necropsied 3, 7, 10, 14, 18, 28 DPV. From each of the necropsied chickens, trachea was collected and fixed by immersion in 10% neutral buffered formalin. Harderian glands were also collected and stored at -85^{o}C until processing.

To compare the amount of immune stimulation/induction between the different vaccine groups, serum for ELISA antibody detection was also collected from individual chickens every 3-4 days 15-28 days post vaccination. RNA was extracted from the Harderian

glands for quantification of immunoglobulin isotype A (IgA) heavy chain mRNA and interferon gamma (IFNγmRNA).

Tracheal histopathology

Formalin fixed sections of trachea were routinely processed, embedded in paraffin, sectioned at 4 to 6 μm, and stained with hematoxylin and eosin for histopathological examination. Individual scores were assigned to each bird for five types of tracheal lesions: deciliation, epithelial necrosis, mononuclear cell infiltration, epithelial hyperplasia, and Goblet cell hyperplasia. The scoring scale was: 1 = normal, 2 = mild lesion, 3 = moderate lesion, 4 = marked lesion, 5 = severe lesion. The significance of differences in mean lesion scores on specific days between groups of chickens inoculated with different vaccines was evaluated by analysis of variance (ANOVA).

The extent of mononuclear cell infiltration was assessed by histomorphometry. The thickness of the mononuclear cell infiltrate layer was measured at five different positions in sections of the cranial one-third of the trachea using ImageJ software (National Institutes of Health, rsb.info.nih.gov/ij/download.html) as described (Toro *et al.,* 2006). The mean for each chicken was calculated and the significance of differences evaluated by ANOVA.

RNA isolation

Total RNA from tears was isolated using the TRI REAGENT® LS - RNA / DNA / PROTEIN ISOLATION REAGENT for LIQUID SAMPLES as per manufacturer's protocol (Molecular Research Center Inc., Cincinnati, Ohio, USA). For each tear sample (30-100 μl) 5 μl of polyacryl carrier (Molecular Research Center Inc.), a compound that helps to precipitate small amounts of RNA, was added. The RNA was resuspended by

addition of 50 µl water, and then stored at -85°C until use. RNA (5 µl) diluted 1:10 was used for quantitative IBV FRET RT-PCR while 5 µl of undiluted RNA was used for conventional RT-PCR.

Harderian gland total RNA was extracted using TRI-REAGENT® RT-RNA/DNA/ PROTEIN ISOLATION REAGENT as outlined by the manufacturer using 50-100 mg of homogenized Harderian gland tissue. RNA was resuspended in 50 µl water and stored at -85°C until use.

Quantification of viral RNA in tears

Viral RNA extracted from tears and further diluted 1:10 as described above was used to determine the IBV viral genome copy numbers in each of the samples by fluorescence resonance energy transfer (FRET)-quantitative reverse-transcription PCR. FRET-qRT-PCR was conducted using a LightCycler (Roche Diagnostics, Indianapolis, Indiana) with primers and probes designed to amplify and detect a portion of the IBV nucleocapsid gene as described (van Ginkel *et al*., 2008). RNA (10^6,10^5,10^4,10^3 copies of in vitro-transcribed RNA) standards as well as negative samples were included in each set of test samples. After the amplification program, melting curves analysis confirmed the specificity of positive reactions.

The calculated viral genomes were converted to the number of genomes/100 µl tears for each sample and further to mean log genomes for each vaccine group. The mean log values were compared using ANOVA.

Conventional RT-PCR and identification of selected viral subpopulations

S1 gene sequencing was done on samples obtained from individual chickens for each vaccine group at 3 and 5 days post vaccination. S1 cDNA for each chicken was prepared from 5 µl of RNA isolated from tears by conventional RT-PCR using the Qiagen OneStep RT PCR kit (Qiagen, Valencia, California, USA). The primers used were NEWS1OLIGO5': 5'TGAAACTGAACAAAAGAC3' (Jackwood *et al.,* 1997) and S1OLIGO3':5'CATAACTAACATAAGGGCAA 3' (Kwon *et al.,* 1993), which amplify a 1.7-kb product of S gene sequence. Where no products were amplified with these primers, alternative primer set NEWS1OLIGO5' and S1R (5'-CATCTGAAAAATTGCCAG-3') (van Santen & Toro, 2008), which amplify an 807-bp product, was used. Products were purified using QIAquick PCR purification protocol (Qiagen, Valencia, California, USA) as per manufacturer's recommendation. The purified products were submitted to Massachusetts General Hospital DNA core facility (Center for Computational and Integrative Biology, Cambridge, MA, USA) for sequencing using S1R primers. Based on the sequences, designations of vaccine subpopulations previously identified in vaccinated chickens (van Santen et al., 2008; Gallardo et al., 2010) were assigned to each sample. All sequence chromatograms were carefully examined independently by two people to identify nucleotide positions with more than one nucleotide, indicating the presence of more than one subpopulation. For such mixed sequences, proportions of each nucleotide were estimated by measuring the height of the major and minor peaks, in order to estimate the proportions of each subpopulation.

Detection of IBV-specific antibodies

IBV specific antibodies in tears diluted 1:10 and serum diluted 1:500 were assayed using a commercial IBV ELISA kit (Idexx Laboratories. Inc., Westbrook, Maine, USA). The sample to positive (S/P) ratios for serum samples were calculated according to kit directions, and serum samples with S/P ratios > 0.2 were considered positive based on the kit directions. The mean S/P ratios for each vaccine group were calculated and compared by repeated measures ANOVA.

IBV RNA, IgA mRNA and IFN mRNA quantification in Harderian gland

To determine relative amounts of IBV RNA, IgA alpha chain mRNA and IFNγ mRNA in Harderian glands of chickens vaccinated with each vaccine and unvaccinated chickens, a two-step reverse transcription (RT) and PCR reaction protocol was used. First strand cDNA was prepared from total Harderian gland RNA according to the SuperSript™III First-Strand Synthesis System for RT-PCR (Invitrogen Life Technologies, CA, USA) protocol. The cDNA for each individual sample was synthesized from 0.2 µg of total RNA using random oligonucleotide primers in a 20 µl reaction volume after which it was stored at -85°C until use. One µl of resulting cDNA (for IgA and IFNγ) and 0.5 µl (for IBV reactions) was used in 20 µl qPCR reactions as described below in a Light cycler ® instrument (Roche, Indianapolis, IN) to quantify cDNA representing IBV RNA or IgA and IFNγ mRNA. The amount of cDNA representing β-actin mRNA in each cDNA preparation was also determined and used to normalize results. The primers to detect the cDNA representing mRNA for the three PCR reactions were designed so genomic DNA would not be amplified.

To quantify IBV RNA in Harderian gland, FRET qPCR was performed on cDNA as described for FRET qRT-PCR in van Ginkel *et al*. (2008) omitting the RT step and using reagents from QuantiTect Probe PCR Kit (Qiagen, Valencia, CA).

IgA mRNA was quantified using a SYBR green qPCR assay designed to amplify a 114-bp section of the chicken IgA heavy chain cDNA (GenBank S40610). The primers used for quantification of IgA mRNA are shown in Table 2.1 and were purchased from Invitrogen®). These primers were designed by Guo *et al*., (2008) (X. Wang, personal communication). The reaction mixture was as described in Huang *et al*. (2001) for qPCR using SYBR green with the following modifications: $MgCl_2$ concentration was increased to 4.5 mM and KCl concentration was increased to 50 mM. The reaction program was the following: initial denaturation 95°C for 5 min; 55 cycles of 94°C for 0 sec; 65°C (used during the first 5 cycles), 63°C (used during the next 7 cycles), 61°C (used during the next 3 cycles) for 12 sec, or 58°C (during the last 40 cycles) for 8 sec; and 72°C for 10 sec. During the last 40 cycles, an additional step, 86°C for 10 sec after the 72°C step, was included to allow for signal acquisition. Melting curve analysis confirmed the specificity of positive reactions.

To determine the relative T-cell response induced in chickens by the different vaccines, IFNγ mRNA in Harderian gland was quantified using an IFNγ FRET qPCR assay. Primers and probes used were as listed in Table 2.1 and were purchased from Operon Biotechnologies, Inc. Alameda, California). These primers amplify and detect a 237 bp fragment of chicken IFNγ cDNA. The reaction mixture was as described (Huang *et al*., 2001) for qPCR using FRET probes. Reaction program includes: initial denaturation step 95°C for 5 min, 43 cycles of 95°C for 10 sec, 65°C (used in the first 6 cycles), 63°C

(used in the next 9 cycles), 61°C (used in the next 3 cycles) or 57°C (used during the last 25 cycles) for 30 sec and 72°C for 30 sec. During the last 25 cycles, an additional step of 50°C for 8 sec was inserted between the 95°C and 57°C steps to allow annealing of the probes. Fluorescence was measured after the annealing of the probes step. As mentioned above, melting curve analysis confirmed the specificity of positive reaction.

For normalization of IBV RNA, and IgA and IFN g mRNA in Harderian gland, a FRET PCR to quantify β-actin mRNA was performed as previously described (O'Neill *et al*., 2009) except that 0.5 µl of cDNA was used in the reaction.

The number of IBV, IgA, and IFNγ mRNA copies quantified in each sample was normalized to β-actin mRNA and the values converted to log values. The normalized mean log values were compiled for each vaccine group and the mean values compared between the groups using ANOVA.

Table 2. 1: *Primers and probes used for quantification of chicken IFNγ and IgA mRNA*

Primer/probe	Sequence
IFNγ1F	5'-GTAGAGAAACTGAAGAACTGGACAGAGAG-3
IFNγ1R	5'-GGCTTTGCGCTGGATTCTCA-3
3' labeled IFNγ Probe	5'-AGGTGAAAGATATCATGGACCTGG (6-Fam-q)-3',
5' labeled IFNγ probe	5'-(AminoC6+B0630)-CAAGCTCCCGATGAACGACTTp-3'
ChIgAF	5'-TGCAGGGCAATGAGTTCGTCTGTA-3'
ChIgAR	5'-AGGAGGTCACTTTGGAGGTGAAT-3'

Results

Viral subpopulations selected in chickens

IBV S1 gene cDNA prepared from IBV RNA extracted from tears of chickens vaccinated with four different Ark serotype vaccines was sequenced to determine viral

subpopulations in vaccinated chickens. The sequenced region encoded the first 250 amino acids of the S1 protein. Differences within this region had previously been found between IBV-Ark vaccines and vaccine subpopulations selected in chickens (Table 2.2). In our previous study, two ArkDPI-derived vaccines (A and C) had been found to contain higher proportions of the selected subpopulations (characterized by nucleotides C and G at S1 gene positions 127 and 637 respectively) than vaccines B and D. We confirmed selection of viral subpopulations as early as three days post-vaccination as previously reported (van Santen & Toro, 2008, McKinley *et al* 2008), in samples from chickens vaccinated with the different ArkDPI vaccines. We found that the subpopulations selected in chickens inoculated with different Ark vaccines differed both in type and frequency (Table 2.3). In chickens vaccinated with vaccine C, all the chickens (100%) had a uniform single subpopulation C2 selected (Table 2.3) which was the major vaccine subpopulation detected in the C vaccine vial previously (van Santen & Toro, 2008). In chickens vaccinated with vaccine A, all the chickens had subpopulation C1 selected. In all the chickens except one, C1 was present as a single apparently uniform subpopulation. A single chicken also the major vaccine subpopulation in addition to C1. In the group of chickens vaccinated with vaccine B, six different subpopulations in addition to the vaccine major population were detected, while in the group that received vaccine D, two different subpopulations were detected (Table 2.3). In the group of chickens that received vaccine B two different subpopulations could often be detected in individual chickens at any one time, unlike the other vaccines where mostly single subpopulations were found. The “selected” subpopulations (based on S1 gene nucleotide positions 127 and 637) were detected in 50% of the chickens vaccinated with vaccine D and in 86% of those

vaccinated with vaccine B. In chickens vaccinated with vaccine B, the selected subpopulations were sometimes found in a mixture with unselected subpopulations (V or C5). In tears, when we compared the incidence of the selected subpopulations between the vaccine groups, we found that the incidence of these subpopulations was significantly higher in chickens vaccinated with vaccine C than D ($p<0.05$) (Fig. 2.1A).

IBV has been shown to replicate locally in the Harderian gland of chicken (Toro *et al*., 1996; van Ginkel *et al*., 2008). We therefore compared viral subpopulations in the Harderian gland in chickens vaccinated with the different vaccines. We found that selected subpopulations were found in significantly higher proportions ($p<0.05$) in chickens vaccinated with vaccine A and C than those vaccinated with vaccine B and D at three days post vaccination (Fig. 2.1B). Thus we found that ArkDPI vaccines A and C resulted in highest incidence of the selected viral subpopulations in vaccinated chickens followed by vaccine B and lastly vaccine D.

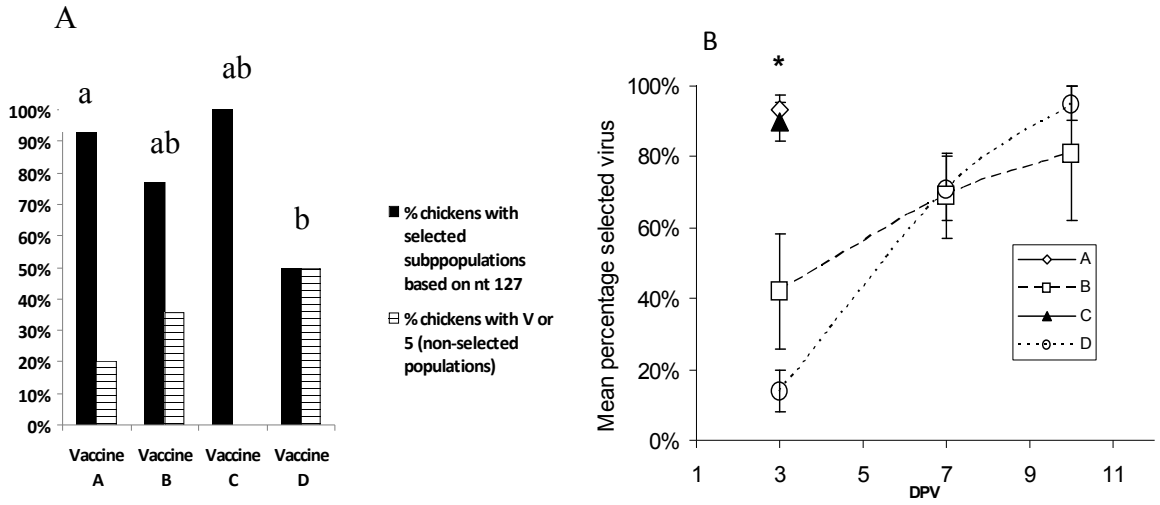

Figure 2. 1: *Incidence of (A) and mean (B) selected viral subpopulations in chickens vaccinated with different ArkDPI vaccines based on selection at nucleotide 127 in tears*

(combined 3 and 5 DPV) and Harderian gland (3,7 and 10 DPV) respectively. Selected subpopulations have a C nucleotide at position 127 of S1 gene unlike the major vaccine subpopulation that has a T at the same position. Based on this criterion, incidence in tears (A) and mean percentage of selected subpopulations in Harderian gland (B) was determined. (A)Data points with different letters are significantly different.(B) The two highest vaccine groups are significantly different from the two other groups on this day. Error bars indicate SEM.*

Table 2. 2: *Viral subpopulations in ArkDPI vaccines and in vaccinated chickens*

		nucleotide position	127	167	226	355	388	511	593	602	637	
		amino acid position	43	56	76	119	130	171	198	201	213	
Major population in vaccine vials[a]:												GenBank accession #
Vaccine A,B,D		nucleotide	T	A	C	T	A	T	A	G	T	EU359638
		amino acid	Tyr	Asn	Leu	Ser	Ser	Tyr	Lys	Gly	Ser	
Vaccine C		nucleotide	**C**	A	C	T	A	T	A	G	**G**	EU359649
		amino acid	**His**	Asn	Leu	Ser	Ser	Tyr	Lys	Gly	**Ala**	
Subpopulations selected in individual vaccinated chickens[b]:												
Designation[c]	**Vaccine[d]**											
1	A, B	nucleotide	**C**[e]	A	C	T	**G**[f]	T	A	G	**G**	EU359643
		amino acid	**His**	Asn	Leu	Ser	**Gly**	Tyr	Lys	Gly	**Ala**	
2	(B), C, D	nucleotide	**C**	A	C	T	A	T	A	G	**G**	EU359650, EU359652
		amino acid	**His**	Asn	Leu	Ser	Ser	Tyr	Lys	Gly	**Ala**	
3	(A, B)	nucleotide	**C**	A	**T**	**C**	A	**C**	**C**	G	**G**	EU359626
		amino acid	**His**	Asn	**Phe**	**Pro**	Ser	**His**	**Thr**	Gly	**Ala**	
4	(A) B	nucleotide	**C**	**G**	C	T	A	T	A	G	**G**	GQ484957
		amino acid	**His**	**Ser**	Leu	Ser	Ser	Tyr	Lys	Gly	**Ala**	
5	(B, D)	nucleotide	T	A	C	T	A	T	A	G	**G**	GQ484958
		amino acid	Tyr	Asn	Leu	Ser	Ser	Tyr	Lys	Gly	**Ala**	
6	(B)	nucleotide	**C**	A	**T**	**C**	A	**C**	A	**A**	**G**	
		amino acid	**His**	Asn	**Phe**	**Pro**	Ser	**His**	Lys	**Glu**	**Ala**	
Consensus among most frequently selected subpopulations (1, 2, 4):												
		nucleotide	**C**	A/**G**	C	T	A/**G**	T	A	G	**G**	
		amino acid	**His**	Asn/**Ser**	Leu	Ser	Ser/**Gly**	Tyr	Lys	Gly	**Ala**	
IBV-Ark-like field isolate consensus:												
		amino acid	**His**	Asn	**Phe**/Leu	Ser/**Pro**	Ser	**His**/Tyr	Lys/**Thr**	Gly	**Ala**	

[a]From van Santen and Toro, 2008

[b]Only subpopulations reproducibly detected in more than one experiment (van Santen & Toro, 2008; Gallardo, *et al*., 2010; present work) are shown.

[c]Subpopulations 1-5 correspond to Components 1-5 in Gallardo, *et al*., 2010.

[d]Vaccine from which the subpopulation is selected. Parentheses indicate that the vaccine subpopulation is only relatively rarely selected from the vaccine indicated.

[e]Black shading indicates difference from vaccine consensus, but same as consensus among most frequently selected subpopulations (1, 2, 4).

[f]Gray shading indicates differences from both vaccine consensus and consensus among most frequently selected subpopulations.

Table 2. 3: *Proportion of chickens with different Ark vaccine viral subpopulations in tears after vaccination with different ArkDPI vaccines at 3 and 5 DPV.*

	#of Sequences	V	C1	C2	C3	C4	C5	C6
Vaccine A	5	1/5(20%)	5/5(100%)	0/5(0%)	0/5(0%)	0/5(0%)	0/5(0%)	0/5(0%)
Vaccine B	14	5/14(36%)	2/14(14%)	2/14(14%)	3/14(21%)	3/14(21%)	3/14(21%)	1/14(7%)
Vaccine C	8	0/8(0%)	0/8(0%)	8/8(100%)	0/8(0%)	0/8(0%)	0/8(0%)	0/8(0%)
Vaccine D	10	3/10(30%)	0/0(0%)	5/10(50%)	0/10(0%)	0/10(0%)	2/10(20%)	0/10(0%)

Viral load in tears and Harderian gland

We quantified the viral loads in tears of chickens vaccinated with four different ArkDPI vaccines using qRT-PCR at 5, 8, 11 and 15 DPV. We detected a 10-fold difference in IBV viral genomes at both day 5 and 8 ($p<0.05$) (Fig. 2.2A) between samples from chickens vaccinated with vaccines with a higher proportion of selected viral subpopulations (A and C) and those with lower proportion (B and D) of selected viral subpopulations. In all the vaccine groups the viral load decreased by day 15. We also compared total viral loads for all days in tears samples found to have the selected subpopulations (based on nucleotides at positions 127 and 637) irrespective of vaccine group and those with the non-selected subpopulations. We found that the viral loads in chickens harboring selected subpopulations were significantly higher at 5 and 8 DPV ($p<0.05$) than those in chickens with other vaccine non-selected subpopulations (Fig. 2.2B). After 8 days post vaccination, most of the chickens only had the selected vaccine subpopulation detected in them indicating that the selected subpopulations may be of higher fitness than other vaccine subpopulations.

To further compare the viral loads associated with different vaccine groups, we quantified the number of IBV RNAs within the Harderian gland total RNA. The viral load dynamics over time followed a similar pattern to that seen in tears above; however we observed no statistically significant differences among the groups (Fig. 2.3). In contrast to results found in tears, in the Harderian glands we also found no relationship between viral load and whether the viral subpopulation was one considered selected.

These results suggest that ArkDPI-derived vaccines with high proportion of selected subpopulation may result in higher viral loads in vaccinated chicken early after infection than those with non-selected subpopulations.

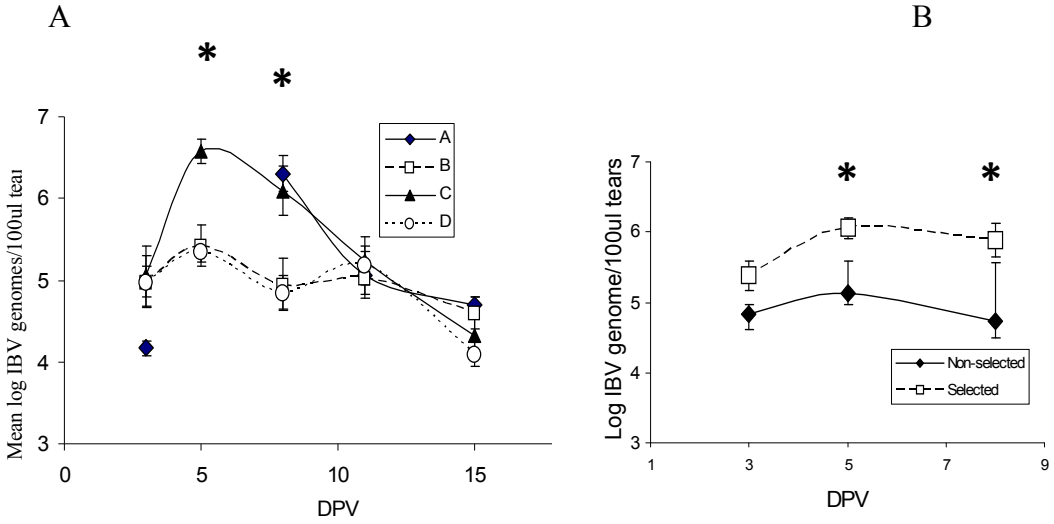

Figure 2. 2: *Viral load in tears (A) Tears were collected from 10 individual chickens vaccinated with the different ArkDPI vaccines, A, B, C, and D on the days indicated post-vaccination. The number of viral genomes in each sample was determined by qRT-PCR. Groups were compared for each day by ANOVA with Tukey's post-test. *Genome values of groups vaccinated with A or C significantly different from B or D. (B) Viral load in samples with selected subpopulation vs. those with non-selected subpopulations. Viral*

*load in tear samples were divided into whether the viral subpopulation was predominantly "selected" or not based on nucleotide at position 127. Viral loads of samples with selected virus were compared to those with unselected virus for each day by Students t-test. * indicate days when viral loads between groups were significantly different ($p<0.05$). Error bars represent SEM.*

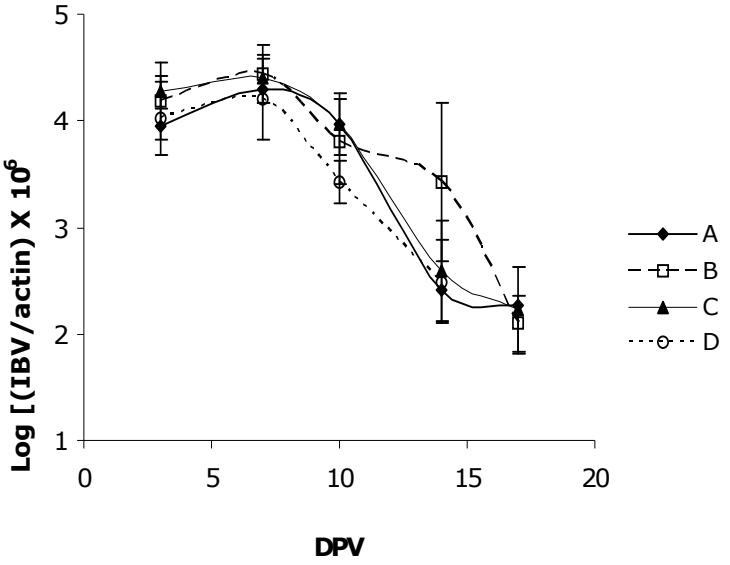

Figure 2. 3: *Six Harderian gland were collected from each vaccine group necropsied chickens for the indicated days and cDNA prepared from Harderian gland total RNA. IBV genomes were determined by quantitative PCR. The number of IBV genomes was normalized to β-actin in each sample. Error bars represent the SEM.*

Respiratory tract vaccine reaction

We compared the incidence of respiratory signs in the different vaccine groups. We found that between 4-7 DPV, different ArkDPI vaccines induced varying incidences of respiratory signs. In general, vaccines previously found to contain a high proportion of selected viral subpopulation (A and C) induced a higher incidence ($P<0.05$) than one that had lower proportion (D) (Fig. 2.4). At 4 DPV chickens given vaccine C had respiratory

signs incidence of 60%, vaccine A 50%, vaccine D 40% and B, 30%. At 4 DPV the trend of incidence of the respiratory sounds was similar to that of viral load seen in tears at 5-8 DPV where vaccine C and A have higher values than vaccine B and D groups. Unexpectedly, chickens vaccinated with vaccine B had incidence of respiratory signs increase from 4 DPV to levels similar to that seen in chickens vaccinated with vaccine A and C. While these results indicate that a higher proportion of vaccine viruses able to efficiently replicate in chickens results in a significantly higher incidence of respiratory signs in vaccinated chickens, the finding of high incidence of respiratory signs in vaccine B group despite low viral load detected suggest that other vaccine inherent factors may play a role in the induction of respiratory signs.

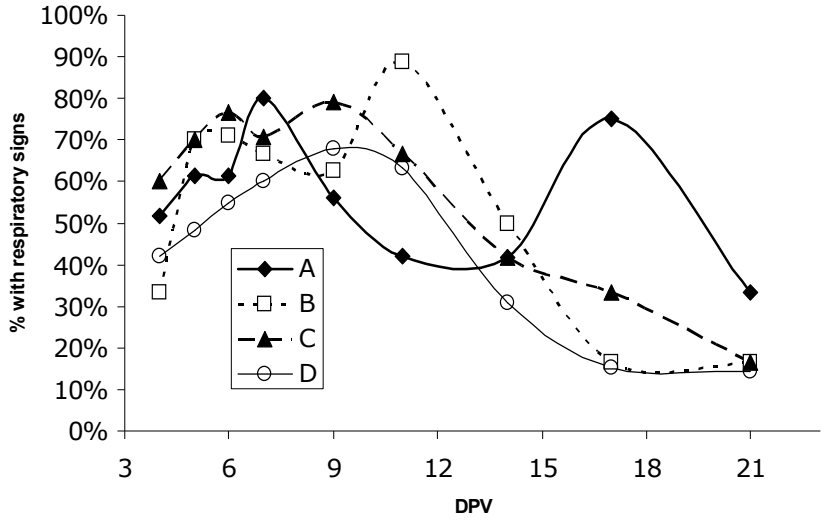

Figure 2. 4: *Incidence of respiratory signs. All chickens in each vaccine group were assessed individually on the days indicated for the presence of respiratory signs. The presence or absence of respiratory signs (tracheal or nasal rales) was determined by bringing the head of each chicken close to the observer's ear. At 4 DPV there were 30 chickens per group. Due to removal of 6 chickens/group for necropsy sampling every 3-4*

days, the number of chickens per group was reduced to 12/group by day 14. Incidence of respiratory signs was compared between groups by Fisher's exact test.

Tracheal histopathology

The severity of vaccine reaction in the different Ark vaccine groups was compared by determining the tracheal lesion scores (necrosis, deciliation, infiltrate, goblet cell hyperplasia and epithelial hyperplasia), and the extent of lymphocytic infiltration. Minimal tracheal histopathology was detected at 3 DPV and no differences were found between the experimental groups. The peak of tracheal histopathology parameters for all vaccine groups was detected at 7 DPV (Fig. 2.5). At 7 DPV chickens vaccinated with vaccine C had a significantly greater thickness of the lymphocytic infiltration layer than the unvaccinated controls and all the other vaccine groups ($p<0.05$) (Fig. 2.5A). We also found that at 7 DPV, vaccine C group of chickens had significantly higher tracheal lesion scores (necrosis, deciliation and infiltrate) than vaccine D chickens (Fig. 2.5 B, C and D respectively). Surprisingly, the lesion scores induced by vaccine B were high and not significantly different from those found in vaccine C group despite having found lower viral load in the tears of these chickens. Furthermore vaccine A group found to have higher viral load also had relatively lower lesions scores (mononuclear infiltrate) than vaccine C chickens ($p<0.05$)

Two peaks for the tracheal lesions and relative thickness of lymphocytic infiltrate were seen in chickens vaccinated with vaccines C and B, one at 7 DPV and another around 14 DPV. Chickens vaccinated with vaccines A and D had one peak (11-14 DPV), which was later than the first peak for chickens vaccinated with vaccines B and C (Fig. 2.5). The other lesion scores assigned and compared between groups included goblet cell and

epithelial hyperplasia. However, most chickens exhibited only mild goblet cell and epithelial hyperplasia and no differences were detected between the different vaccine groups (data not shown). These results indicate that the severity of the vaccine reaction in the trachea after administration of different ArkDPI vaccines differs among the different Ark vaccines. In general, at 7 DPV the vaccines that have a high proportion of selected subpopulation induce more severe tracheal lesions than those that have lower proportions of selected subpopulations.

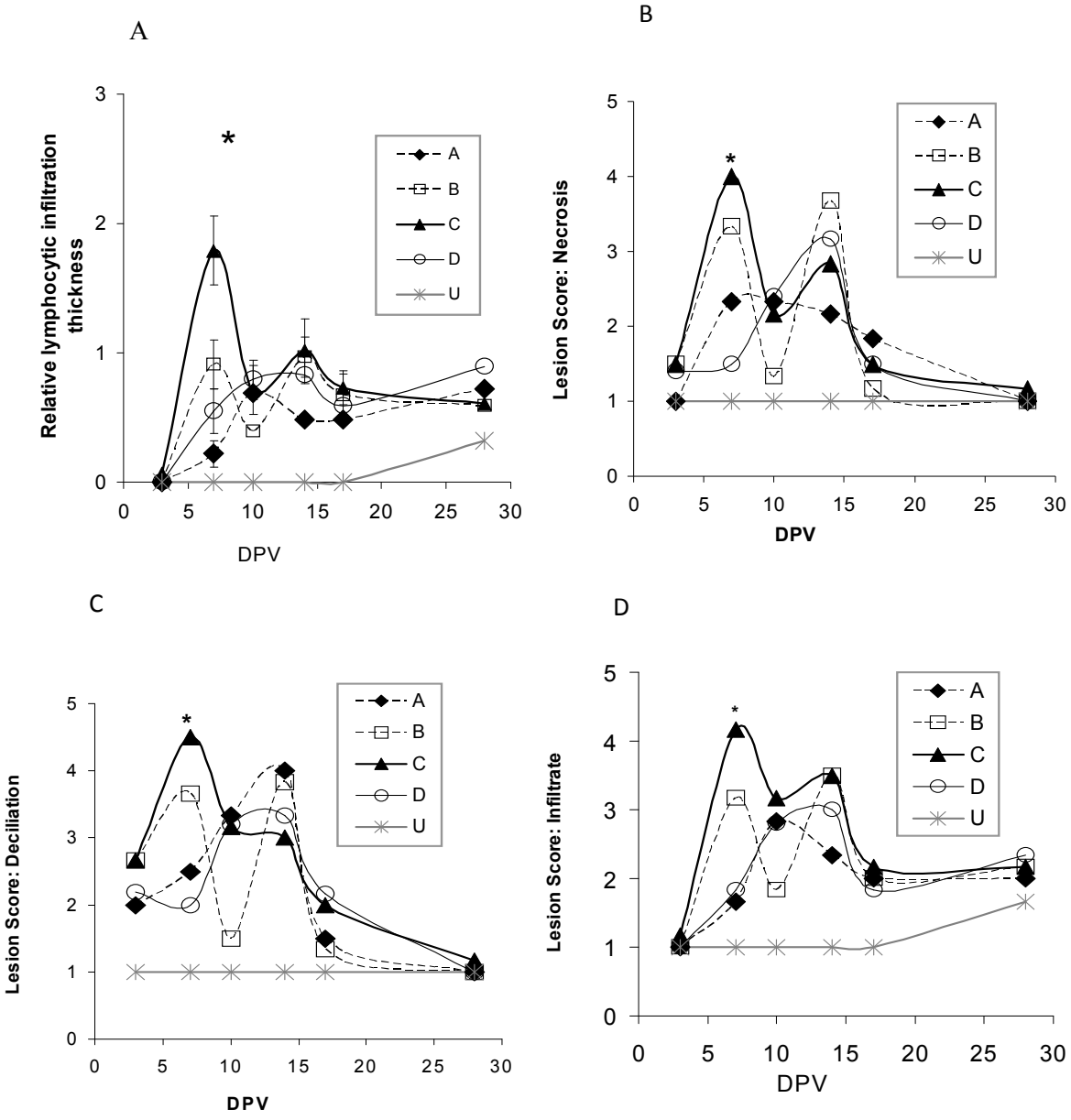

Figure 2. 5: *Trachea histopathology. Six chickens in each vaccine group were necropsied on the days post-vaccination indicated and trachea samples routinely processed for histopathological examination. (A) The thickness of lymphocytic infiltration into the tracheal mucosa was measured at 5 points for each sample. Means were calculated for each vaccine group and compared. *The lymphocytic infiltrate was significantly more intense for vaccine group C 7 DPV than for all other groups. (B), (C),*

*and (D) Lesion scores from 1 (normal) to 5 (severe) for necrosis (B) deciliation (C) and mononuclear infiltrate (D) were assigned. *Lesion scores for vaccine group C 7 DPV were significantly higher ($P<0.05$) than vaccine group D.*

IBV specific antibodies in serum

Serum collected from vaccinated chickens 15-27 DPV was analyzed for total IBV specific antibodies. The pattern of induction of serum antibodies was similar whether we looked at incidence of positive chickens based on the kit cut off or compared ELISA values in form of S/P ratios (Fig. 2.6). The incidence of positive chickens started at a low of 20% by 15 DPV to a peak around 24-28 days for all the vaccine groups. The incidence of positive chickens at peak and mean S/P ratio was higher for the chickens that received vaccines A or C than those that received vaccine B or D between 21-28 DPV (Fig. 2.6A). We compared the magnitude of the serum IBV specific antibodies induction by different ArkDPI vaccines using repeated measures ANOVA. Vaccine A induced the highest amount of IBV specific antibodies followed by vaccine C, D and B (Fig. 2.6B). One of the vaccines with a higher proportion of selected viral subpopulation (A) induced significantly higher ($P<0.05$) IBV specific antibody than those with lower proportion (B and D).

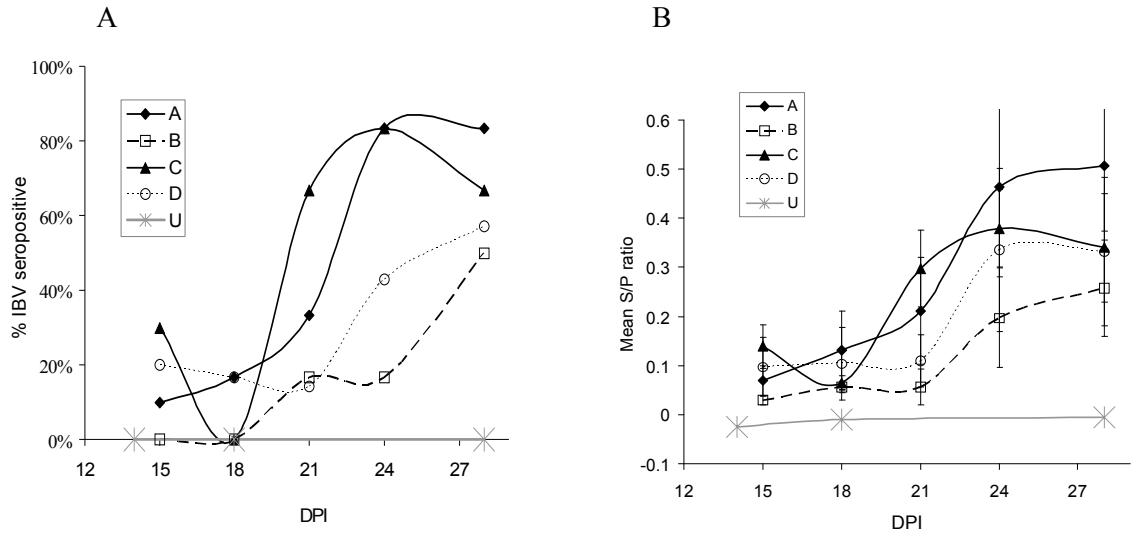

Figure 2. 6: *Detection of IBV specific antibodies in serum of Ark serotypes vaccinated chickens using an ELISA assay. Serum collected from vaccinated chickens 15-27 DPV was analyzed by ELISA using a commercial IDEXX IBV kit. A) S/P ratios were calculated based on the kit formula and the number of samples with an S/P ratio >0.2 were tabulated for each vaccine. B) Mean S/P ratios calculated for each vaccine group Error bars represent SEM*

IgA and IFN mRNA in Harderian gland

We determined the relative amount of IgA mRNA in the local lymphoid organ (Harderian gland) using a SYBR green quantitative assay and normalized values to β-actin mRNA. IgA mRNA was not detectable in most samples at 7 DPV. Chickens in all vaccinated groups had significantly higher IgA mRNA than uninfected controls 10 -14 DPV. When we compared the chickens in the different vaccine groups, we found that chickens that received vaccine A had significantly higher (*P=0.05*) magnitude of IgA mRNA induction at 10 and 14 DPV than those that received vaccines B or D (Fig. 2.7).

Although few significant differences between groups for each sampling day were detected, in general vaccines with a higher proportion of selected viral subpopulation had higher levels of IgA mRNA (vaccines A and C vs. B and D) (Fig. 2.7A).

We also compared IFNγ mRNA induction in the Harderian gland between the chickens receiving the different vaccines as another measure of immune induction. The IFNγ mRNA values from each chicken were normalized to β-actin mRNA values for the same chicken and the mean log values for each vaccine group calculated and compared. In contrast to differences in IgA mRNA detected between groups, differences in IFNγ mRNA were not noted. Instead, the different vaccines induced a similar IFNγ mRNA pattern with a peak at day 10 followed by steady decline (Fig. 2.7 B).

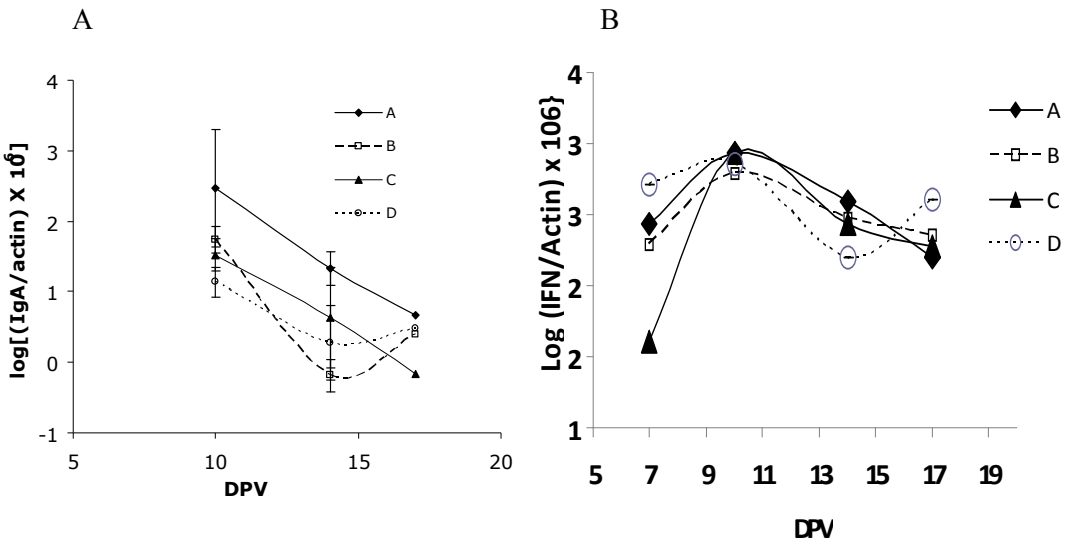

Figure 2. 7: *Immune response gene expression in Harderian glands. Six chickens in each vaccine group were necropsied on the days indicated post-vaccination. RNA extracted from Harderian glands of individual chickens was converted to cDNA using random primers and the level of IgA heavy chain (A) and IFNγ (B) cDNA quantitatively determined. Results were normalized to ß-actin cDNA levels. Error bars indicate SEM.*

Discussion

Recent concerns about the predominance of field IBV isolates having S1 sequences related to the ArkDPI molecular type have raised interest in the Ark serotype vaccine characteristics both before and after administration to chickens (Nix *et al.,* 2000; Jackwood *et al.,* 2005 & 2009; Toro *et al.,* 2006). Subsequent studies have demonstrated that commercially available live attenuated ArkDPI derived vaccines are heterogeneous in nature (Nix *et al.,* 2000; van Santen & Toro, 2008; McKinley *et al*., 2008) and also differ in the amount of heterogeneity (van Santen & Toro*,* 2008). After a single passage in chickens, some Ark vaccine subpopulations were selected as early as three days post vaccination, which may point to a possible difference in the relative fitness of the vaccine viral subpopulations. Differing proportions of these selected subpopulations were also found in the different ArkDPI vaccines (van Santen & Toro, 2008).

In this study, we have looked at the significance of the presence of different proportions of the selected vaccine subpopulations amongst the ArkDPI vaccines as related to viral replication in vaccinated chickens. Our study indicated that vaccines that have a high proportion of selected subpopulations resulted in significantly higher viral loads in tears up to 8 DPV in vaccinated chickens than the vaccines with lower proportions. This observation may point to a difference in the replication ability of the different ArkDPI vaccines in the ocular tissues. Most vaccines administered by ocular routes including IBV initially replicate in the epithelial cells of eye before spreading to other tissues in the body (Toro *et al*., 1996; Russell, 1993). It is thus important that vaccines be able to replicate in the eyes to titers that can spread to respiratory tissue including the trachea for effective immune responses to be induced in these tissues.

Furthermore research has shown that ocular IBV infection results in IBV-specific neutralizing antibodies in tears, which may be important although not solely responsible for protection (Gelb *et al.,* 1998; Cavanagh, 2007; Payne, 1994; Davelaar *et al*., 1982; Davelaar & Kouwenhoven, 1982). Failure of vaccine viruses to replicate in the eye may therefore result in failure of vaccine to induce protective immune response. Excessive replication on the other hand may also mean that the vaccine is not well attenuated and may result in excessive pathology in the respiratory tract, the sequel of which is susceptibility of chickens to other opportunistic infections. This balance in vaccine development is of concern with live attenuated vaccines (Bijlenga *et al*., 2004).

We found that after an initial peak by 8 DPV in viral load in chickens vaccinated with vaccines harboring high proportions of selected subpopulation (A and C), a sharp decline occurred by 15 DPV. This was a sharp contrast to the viral loads detected in chickens vaccinated with two other vaccines that had low amounts of the selected subpopulations. The viral load of these vccines did not have a clear peak; neither was there a sharp decrease through to the last day of analysis. In addition, the group of chickens vaccinated with vaccine B showed a higher viral load than the other vaccine groups at the last day of sampling which indicated that no significant control of viral replication occurred from the day of inoculation to the last day of sampling. It is possible that the low viral copies of selected subpopulations present in the vaccine B did not stimulate the immune response strongly enough to result in elimination of vaccine viruses as fast as seen in A and C groups but rather resulted in vaccine viruses being maintained in the chicken at low levels but for a longer time. Persistence of vaccine viruses long in vaccinated chickens may have some negative consequences as they may undergo

mutations and /or recombination in the field resulting in generation of more virulent variants (van Santen & Toro, 2008). In addition to inducing higher viral loads we found that the same vaccines with higher proportion of selectable viral subpopulations induced a higher incidence of respiratory signs and tracheal pathology (C) than vaccine with lower amounts (D) at 7 DPV, indicating a more severe vaccine reaction in the respiratory tract. One of the vaccines previously found to have very low amounts of selected subpopulations (vaccine B) unexpectedly showed a high incidence of respiratory signs and also significantly higher tracheal vaccine reaction similar to vaccine C vaccinated chickens. Respiratory sounds (rales) and tracheal lesions detected in infected /vaccinated chickens arise as result of viral replication in upper respiratory tract especially the upper third of trachea where the IBV replicates (Geilhausen *et al*., 1973). One explanation for this observation with this Ark vaccine may be that vaccine B, though having a smaller proportion of selected subpopulations, has other subpopulations more fit for the upper respiratory system that are selected over time to cause this increase in the respiratory reaction. Additionally, the low viral load detected in chickens vaccinated with this vaccine may result in lower induction of immune response further favoring vaccine viruses with a tracheal tropism to replicate and cause more pathology without clearance. Tracheal histopathology findings further strengthen this hypothesis since we found lesion scores associated with this vaccine to follow a similar pattern as the incidence of respiratory signs.

Induction of protective immune response is the main goal of vaccination. Our study revealed that the serum IBV-specific antibodies were higher and appeared earlier in chickens vaccinated with vaccines A and C than B and D. Local antibody response as

assessed by IgA mRNA in the Harderian gland, although not demonstrated to be IBV-specific, was also found to be higher in chickens vaccinated with vaccines A and C .We found the four vaccines to induce IFNγ production at similar levels.

Our study reveals important differences in the outcome of vaccination by ArkDPI-derived Ark serotype IBV vaccines containing different viral subpopulation characteristics. We have found that these differences can be explained in part by the inherent differences in vaccine subpopulation structures especially the proportion of vaccine viruses able to efficiently replicate in the upper respiratory tract of chicken. Further research on these specific vaccine subpopulations in their cloned state may shed more light on their ability to protect, induce pathology or persist in vaccinated chickens.

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CHAPTER 3

IMMUNE RESPONSE, RESPIRATORY TRACT PERSISTENCE AND VIRAL LOAD AFTER EXPERIMENTAL VACCINATION WITH TWO DIFFERENT ARK VACCINES

Abstract

Factors responsible for the high frequency of isolation of ArkDPI viruses in respiratory cases of confirmed IBV are still unclear. The inherent characteristics of ArkDPI vaccines marked by presence of viral subpopulations that efficiently replicate in the upper respiratory tract of chicken may play a role. The present study compared the vaccination outcome of two high titered ArkDPI vaccines, one with a high proportion of the selected subpopulation (A) and another vaccine with a lower proportion of selected subpopulations but was previously found to result in mixed subpopulations being established in different chickens (B). The number of viral subpopulations detected per chicken was significantly higher in chickens vaccinated with vaccine B than A. While IBV was detected in the tears of all chickens vaccinated by either vaccine, those chickens vaccinated with vaccine B had significantly higher viral load than those vaccinated with vaccine A. Vaccine B also induced a significantly higher lachrymal IgM response, higher serum antibodies and an earlier peak of IBV-specific IgA.

However vaccine B also resulted in a significantly higher number of birds with IBV virus in the trachea at 20 DPV than vaccine A. Interestingly despite detecting multiple subpopulations in the chickens vaccinated with this vaccine at 5 and 7 DPV, a single subpopulation was found to persist at 20 DPV in trachea. This finding of differences in persistence of Ark vaccine viruses in the trachea is significant and warrants further analysis of these vaccine subpopulations.

Introduction

With the use of live attenuated vaccines to control disease, the balance between sufficient replication to levels that induce protective immunity and ability to cause pathology is a subject of concern (Bijlenga *et al*., 2004). While live attenuated vaccines are routinely checked for reduced virulence before release for use in animals, concern still exists on the stability of the vaccine viruses, in particular reversion to virulence when administered to the natural host or presence of residual virulent subpopulations within the vaccines (Brugh, 1988; Muskett *et al*., 1985; Naylor & Jones, 1994). Use of live attenuated vaccines in chickens amounts to back-passaging and so depending on the stability of the vaccines, generation/selection of more virulent variants can occur, resulting in pathology in vaccinated subjects (Guy *et al*., 1991; Hopkins & Yoder, 1984, 1986). Vaccine viruses that persist long in the field after vaccination can also serve as source of genetic material for mutations and recombination (McKinley *et al*., 2008; van Santen & Toro, 2008).

Avian infectious bronchitis is a highly infectious respiratory disease that continues to be of economic importance worldwide (Cavanagh, 2007). The disease is caused by infectious bronchitis virus (IBV), a single-stranded, positive RNA coronavirus belonging to the group 3 coronaviruses (Cavanagh *et al*., 2002). Infection in young chicks

results in increased mortality and reduced weight gain accompanied by post slaughter carcass condemnation in surviving meat birds. In laying birds, signs include reduced egg production, condemnation of eggs due to deformations and poor eggshell and internal quality **(**Cavanagh, 2005, Britton & Cavanagh, 2007**)**. Control has been through the use of vaccines, which has reduced the losses by tremendous magnitudes. However despite these gains, the disease continues to be ranked among the most important disease affecting chickens in the US and Europe (Jackwood *et al*., 2005; Bijlenga *et al*., 2004, Cavanagh & Naqi, 2003; Cavanagh, 2007). Immunity to IBV results after natural infection and vaccination but many studies report no association between the amount of serum antibodies and protection (reviewed in Cavanagh, 2008). Some authors however have found association of lachrymal IBV-specific IgA and protection against IBV (Toro & Fernandez, 1994; Cook *et al*., 1992) and suggested IgA in tears as good measure of protection. IBV exists as several serotypes that poorly cross protect. Rapid and unpredictable appearance of new serotypes occurs in the field, which is thought to be through spontaneous mutations and/or recombination (Cavanagh *et al*., 1992; Jia *et al*., 1995; Cavanagh, 2005; Bijlenga *et al*., 2004).

In the US, six different IBV vaccine serotypes are routinely used in flock vaccinations with the Massachusetts, Arkansas and the Connecticut serotypes being the most commonly used (Jackwood *et al*., 2005). Despite extensive vaccinations, Arkansas-like (ArkDPI, Ark99, and other "Ark-like" genotypes) viruses continue to be the most commonly isolated viruses from respiratory cases confirmed of IBV in commercial broiler flocks in the southeastern US (Jackwood *et al*., 2005, 2009; Toro *et al*., 2006; Nix *et al*., 2000). Recent field studies have indicated that after vaccination with combinations

of prevailing serotypes, the Arkansas-type viruses persisted longer than the other serotypes in the chickens (Alvarado *et al*., 2006; Jackwood *et al*., 2009) and that the degree of protection against Arkansas challenge strain was lower (37.5-65%) than protection against another serotype (Delaware, 100%) after vaccination with a combined regimen that included both serotypes (Jackwood *et al*., 2009). The factors responsible for the increased frequency of Ark-like isolations and the seemingly low protection rates are not clearly understood. Among others, there are concerns regarding the stability of the Ark-type vaccines and possibility of reversion to virulence once administered to chickens. Studies have indicated that commercially available Ark serotype IBV vaccines from different manufacturers (van Santen & Toro, 2008; McKinley *et al*., 2008) and different productions stocks (McKinley *et al*., 2008) exhibit heterogeneity in their viral subpopulation structures despite being from the same seed stock. In addition, some minor viral subpopulations found in these vaccines were found to apparently replicate better in vaccinated birds and were selected by three days post-vaccination. These selected viral subpopulations appeared to be in the parent vaccines in differing proportions before being administered to the chickens (van Santen & Toro, 2008). These differing proportions of selectable virus were found to affect viral load, incidence of respiratory signs and tracheal histopathology and systemic immune response in experimentally vaccinated chickens (Chapter 2). In general, ArkDPI-derived vaccines with higher proportions of selectable virus induced significantly higher viral loads, incidence of respiratory signs, tracheal lesions and systemic immune response. Additionally, IBV Ark-DPI derived vaccines from different manufacturers resulted in differing viral subpopulations selected in vaccinated chickens. While some of the vaccines resulted in a predominantly uniform

subpopulation selected in vaccinated chickens, one vaccine resulted in selection of multiple subpopulations and sometimes more than one subpopulation present in individual chickens at any given time (van Santen & Toro, 2008; Gallardo *et al*., 2010). Presence of viral subpopulations has been reported for other poultry vaccines and wild type viruses too. Chicken infectious laryngotracheitis virus (ILTV) vaccines, infectious bursal disease virus (IBDV) vaccines and mildly pathogenic H5N2 avian influenza viruses have all been reported to have multiple viral subpopulations (Garcia & Riblet, 2001; Jackwood & Sommer, 2002; Brugh & Perdue, 1991). It has been suggested that vaccine virus heterogeneity may be responsible for the broader immune response induced by some IBV vaccines (McKinley *et al*., 2008) and may be beneficial (Nix *et al*., 2000), but concerns also exist that this heterogeneity may offer genetic material for generation of new variants by recombination and mutations (van Santen & Toro, 2008; Nix *et al*., 2000). In viral evolution in the host, presence of a diverse pool of viral genetic subpopulations may be beneficial to the virus by allowing rapid adaptation to the host through selection of the best-fit virus (Domingo *et al*., 1998). Existence of several viral subpopulations may also enable the virus to infect a broad spectrum of tissues within the host through cooperative interactions (Vignuzzi *et al*., 2005). An expanded tissue/cell tropism within the infected host may have an effect on the magnitude of pathology and/or induced immune response in the infected host and/or persistence of viruses within the host (Domingo & Gomez, 2007). In this study we used two different Ark IBV vaccines that inherently differ in their proportion of selectable virus that we previously demonstrated induced differing viral loads and systemic immune responses (Chapter 2). In addition one of the vaccines had resulted in a pool of heterogeneous viral

subpopulations in vaccinated chickens. The objectives of this study therefore were compare the: a) viral load in tears b) mucosal IgA, IgM and systemic immune responses and c) viral subpopulation dynamics over time in tears, trachea and cloacae. This information further helps to understand IBV vaccine dynamics in vaccinated birds and provides information useful for designing more efficacious and safer vaccines.

Materials and Methods

Experimental design

Two single entity commercial live-attenuated IBV Ark-serotype vaccines from different manufacturers originally derived from the Ark-DPI strain were used in this study. The two vaccines were chosen based on their proportion of selectable virus and previous characteristics after a single passage in chickens. Vaccine A had a higher proportion of selectable virus and induced a higher systemic immune response. Vaccine A also had resulted in a predominantly single viral subpopulation in chickens, while vaccine B had no predominant subpopulation selected but rather up to 5 different subpopulations detected in individual vaccinated chickens (Chapter 2, van Santen & Toro, 2008). The vaccines were coded A and B corresponding to the same codes used in the previous study. Each bird received 1.5 X 10^5 50% embryo infectious doses (EID_{50}) divided equally in both eyes and nares.

Each vaccine group had 16 specific pathogen free (SPF) chickens. Chickens were wing banded and vaccinated at 12 weeks of age. Each group of birds receiving the same vaccine was housed in separate rooms. Absolute care was taken between sampling from groups administered different vaccines to ensure the groups were not cross-contaminated.

Tears from individual chickens (50-200 µl) in each vaccine group were collected prior to vaccination and 3, 5, 8, 11, 14, 17 and 21 DPV as described (Toro *et al*., 1993) and immediately cooled to 4° C in ice until they were taken to the laboratory where they were stored at -20° C. Between 3 and 11 DPV, tears were collected from alternating eyes, thus 3 and 8 DPV were from the same eye and 5 and 11 DPV were from the same eye. Tears were used for IBV RNA extraction and also analyzed by ELISA for IBV-specific IgA and IgM. RNA extracted from tears was used for viral load quantification using quantitative RT-PCR and also conventional RT-PCR to amplify the IBV spike gene (S1 subunit) cDNA for sequencing.

To determine the presence of vaccine viruses and viral subpopulations in other tissues, tracheal and cloacal swabs were taken from birds at 5, 7 and 20 days post vaccination. The swabs were placed in 2 ml of tryptose phosphate broth, transported in ice and stored at -85° C until RNA extraction. The RNA was used for RT-PCR to generate S1 cDNA for sequencing.

Whole blood (approximately 1.5 ml) was collected from vaccinated chickens before vaccination (pre-immune serum), 5, 8, 11, 17, 21, 24 and 28 DPV. Sera extracted from the blood were used for IBV-specific total antibody determination.

RNA isolation

Total RNA from tears, tracheal and cloacal swabs was isolated using the TRI REAGENT® LS - RNA / DNA / PROTEIN ISOLATION REAGENT for LIQUID SAMPLES as per manufacturer's protocol (Molecular Research Center Inc., Cincinnati, Ohio, USA). For each tear sample (30-100 µl), tracheal and cloacal swabs (250 µl)), 5µl

of polyacryl carrier (Molecular Research Center Inc.), was added to facilitate precipitation of low concentrations of RNA. The RNA was resuspended by addition of 50 µl water, and then stored at -85° C until use. 5 µl of RNA per reaction was used for detection and quantification of IBV RNA.

Quantification of viral RNA in tears by quantitative RT-PCR

5 µl of viral RNA extracted from tears was used to determine the IBV genome copy numbers in each of the tear samples by fluorescence resonance energy transfer (FRET) - quantitative reverse-transcriptase (RT)-PCR. FRET-qRT- PCR was conducted using a LightCycler (Roche Diagnostics, Indianapolis, Indiana) with primers and probes designed to amplify and detect a portion of the Ark IBV nucleocapsid gene as previously described (van Ginkel *et al*., 2008). RNA (10^6,10^5,10^4,10^3 copies of in vitro-transcribed RNA) standards as well as negative samples were included in each set of test samples. After the amplification program, melting curves analysis confirmed the specificity of positive reactions. The calculated viral genomes were converted to log genomes/per 100 µl of tears for each sample.

Conventional RT-PCR and identification of selected viral subpopulation

S1 cDNA for each chicken was prepared from 5 µl of RNA isolated from tears by conventional RT-PCR using the Qiagen OneStep RT PCR kit in a 50 µl reaction. The primers used were (NEWS1OLIGO-5'TGAAACTGAACAAAAGAC 3' and S1OLIGO-5'CATAAACTAAACATAAGGGCAA 3') that amplify a 1.7-kb product of S gene sequence. Products were detected by gel electrophoresis followed by SYBR green fluorescent DNA gel stain. For samples where no products were seen, RT-PCR was repeated using primers NEWS1OLIGO and S1R (van Santen and Toro, 2008), which

amplify a 807-bp S1 gene sequence or S17F and S18R primers (Gallardo *et al.,* 2010), which amplify a 750-bp segment in the S1 gene. Sequencing of a portion of the amplified region was used to differentiate the different viral subpopulation found in vaccinated chickens. RT-PCR products were purified using QIAquick PCR purification protocol (Qiagen, Valencia, California, USA) as per manufacturer's recommendation. The purified products were submitted to Massachusetts General Hospital DNA core facility (Center for Computational and Integrative Biology, Cambridge, MA, USA) for sequencing using S1R primers. Based on the sequences, designations of vaccine subpopulations previously identified in vaccinated chickens (van Santen *et al*., 2008; Gallardo *et al*., 2010; Table 2.2, Chapter 2) were assigned to each sample. All sequence chromatograms were carefully examined independently by two people to identify nucleotide positions with more than one nucleotide, indicating the presence of more than one subpopulation. For such mixed sequences, proportions of each nucleotide were estimated by measuring the height of the major and minor peaks, in order to estimate the proportions of each subpopulation.

Detection of IBV-specific antibodies

IBV-specific antibodies in tears diluted 1:10 and serum diluted 1:500 were detected using a commercial IBV ELISA kit (Idexx Laboratories. Inc., Westbrook, Maine, USA). For serum samples the sample to positive (S/P) ratios were calculated according to the kit directions, and serum samples with S/P ratios > 0.2 were considered positive based on the kit directions. The mean S/P ratios for each vaccine group were calculated and compared by Student's t test. For detection of IBV specific IgA in tears, the protocol was modified as previously described (Toro *et al*., 2006). Similarly, for specific IBV IgM in tears

diluted 1:10, commercial IBV ELISA kit was used with substitution of the horseradish peroxidase conjugated goat anti-chicken IgM (Bethyl Lab., Inc., Montgomery, Texas, USA) for the conjugated kit immunoglobulin.

Results

Viral subpopulations selected in tears of vaccinated chickens

Viral subpopulations in tears collected from each vaccinated chicken at 3, 5, 8 and 11 DPV were determined. IBV RNA was detected by RT-PCR in RNA prepared from tears of each of the vaccinated chickens at 3, 5 and 8 DPV irrespective of the vaccine (A or B) administered. Viral subpopulations were differentiated and classified as C1-C5 based on nucleotide polymorphisms at positions 127, 167, 226, 355, 388, 511, 593 and 637 of the S1 gene as previously described (Gallardo *et al*., 2010). We collected tear samples from alternating eyes between 3 and 11 DPV. Hence samples collected at 3 and 8 days are from the same eye and 5 and 11 DPV from same eye. In a separate experiment, analysis of samples collected from both eyes over time indicated independent selection and maintenance of vaccine subpopulations in separate eyes of the same chicken (V.L. van Santen, unpublished results). Therefore subpopulations found at 3 and 5 DPV were combined to reflect those in both eyes at early times post-vaccination and subpopulations found 8 and 11 DPV combined to reflect those in both eyes at later times. We found that chickens vaccinated with vaccines A or B had different subpopulation structures both at 3 and 5 and at 8 and 11 DPV (Table 3.1). While 100% of chickens vaccinated with vaccine B had at least two viral subpopulations in individual chickens at 3 and 5 DPV, chickens vaccinated with vaccine A had a predominantly single subpopulation in 62.5% of the chickens during the same period. Multiple subpopulations were still present in individual

samples from chickens vaccinated with vaccine B at 8 and 11 DPV (Table 3.1). In addition to the six populations shown in Table 2.2, four additional variants were each identified two as the single IBV population in two samples at 11 DPV and the other two in mixed subpopulation in tears and trachea. Two variants each differed from the C3 subpopulation in one amino acid position, one variant differed from population C5 in two positions, one from subpopulation C3 in two positions. Consequently, each of these variant subpopulations was labeled after subpopulation to which it was most similar with a lowercase v added (i.e. C3v and C5v) in Tables 3.1 and 3.2. We compiled the total number of different subpopulations detected in each chicken for all days sequenced, calculated the means for both groups, and compared the mean number of subpopulations in individual chickens between the two vaccine groups by Mann Whitney. Chickens vaccinated with vaccine B had a significantly higher (*P=0.0004*) number of subpopulations than those vaccinated with vaccine A (Fig. 3.1).

When viral subpopulations in individual eyes were analyzed on subsequent days in the group with most subpopulations per chicken (vaccine B group), we found that some of the chickens that had mixed subpopulations at day 3 lost some of them by day 8. Some subpopulations appeared to be less fit to compete especially when they were initially found in mixed subpopulation. Specifically, where a chicken had C5 or the vaccine major population in combination with any of the other subpopulations (C1, C2, C3 or C4) at 3 DPV, C5 and vaccine major became the minor component or absent by 8 DPV (Table 3.2). However where C5 existed alone in an individual chicken, it was still detectable up to 11 DPV in these chickens suggesting that competition between the various subpopulations was the main factor determining selection (days 5 and 11,Table 3.2).

These results suggest that IBV Ark vaccines result in different viral subpopulation structures in vaccinated chickens. Also heterogeneous viral subpopulations in vaccinated chicken may undergo temporal viral evolution/selection in the vaccinated chickens.

Table 3. 1: *Viral subpopulations detected in tears of chickens*

Vaccine A	3 & 5	8 &11	Vaccine B	3 & 5	8 &11
A1	C1,C4	C4	B1	C1,C5	C1
A2	C1,C4	C1	B2	C2,C3,C4	C4,C5
A3	C1,C5	C1	B3	C1,C4,C5	C1,C4,C5v
A4	C1	C1	B4	C1,C4	C1,C4
A5	C1,C3	C1	B5	C1,C4,C3v	C2,C4
A6	C1	C1,C3	B6	C3,C4	C4
A7	C1	C1	B7	C2,C3,C4	C1,C3v
A8	C1	C1	B8	V,C4,C5	C5
A9	C1	C1,C4	B9	C2,C3,C4	C1,C3,C4
A10	C1	C1	B10	C1,C5	C1
A11	C1	C1	B11	C2,C4,C5	C5
A12	C1	C1	B12	V,C3,C4	C4,C5v
A13	C1,C4	C1,C4	B13	C1,C4	C1,C4
A14	C1,C3	C1,C4	B14	C3,C4	C3,C4
A15	C1	C1,C4	B15	V,C1	C4
A16	C1	C1	B16	C1,C4	C1

Table 3. 2: *Viral subpopulations in vaccine B group at 3, 5, 8, and 11 DPV*

Chk #	3	8	5	11
1	C1,C5	C1		
2	C3,C4	C4,C5	C2,C4	C5
3	C1,C4	C1,C4	C5	C5v
4	C1,C4	C1,C4	C4	C4
5	C1,C4	C2	C3v	C4
6	C4	C4	C3	
7	C2,C3	C1	C4	C3v
8	V,C5	C5	C4	
9	C2,C3	C1,C3	C4	C4
10	C1	C1	C5	
11	C2,C4	C5	C5	C5
12	C3,C4	C4	V,C3	C5v
13	C4	C4	C1	C1
14	C4	C4	C2	C3
15	C1	C4	V	
16	C1	C1	C4	

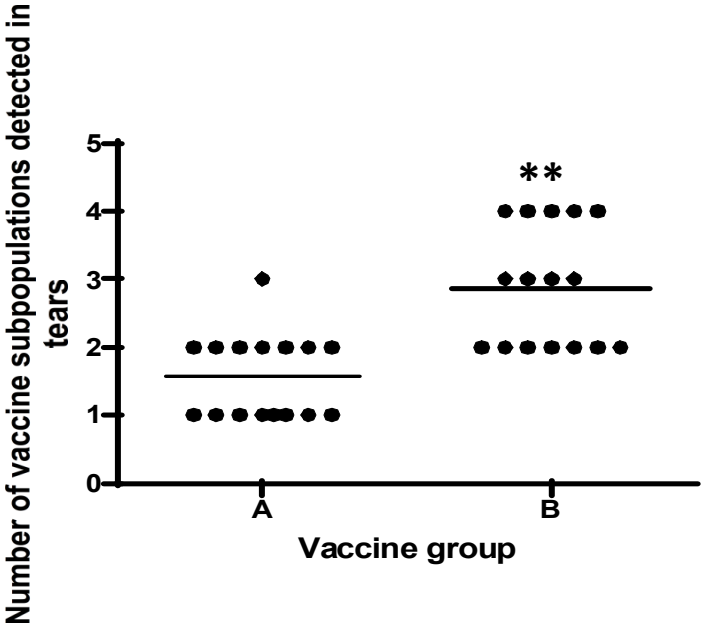

Figure 3. 1: *Number of subpopulations/chicken in vaccinated chickens. Subpopulations were determined from sequence chromatograms of amplified IBV S gene cDNA prepared*

*from RNA extracted from tears collected from alternating eyes 3, 5, 8, and 11 DPV from chickens vaccinated with vaccine A and B and the number total number of subpopulations detected in all samples collected from each chicken complied. Number of samples for each chicken is shown on a scatter plot. The horizontal lines indicate the mean number of subpopulations per chicken for each vaccine group. **Using Mann Whitney and T test, the two means are significantly different (P=0.0004).*

Viral load and incidence of detection in tears

Different Ark vaccine subpopulations may have different replication efficiency in the ocular tissues. We compared the viral load in chickens vaccinated with the two Ark serotype IBV vaccines. No significant differences in viral load were found between the two groups of chickens 3 DPV. However by 5 DPV, chickens vaccinated with vaccine B had significantly higher ($P<0.05$) viral load than those vaccinated with vaccine A (Fig. 3. 2). At 8 and 11 DPV, the viral load in chickens vaccinated with vaccine B remained higher although not statistically significantly different from vaccine A.

Comparing the incidence of IBV positive chickens, at 3, 5 and 8 DPV, all chickens were positive for IBV virus detection by qRT-PCR irrespective of the vaccine group. By 11 DPV, the incidence of positive vaccine virus detection in vaccine group A was markedly reduced (25%) as compared to vaccine group B (50%) indicating slower viral clearance for chickens vaccinated with vaccine B than A (not shown).

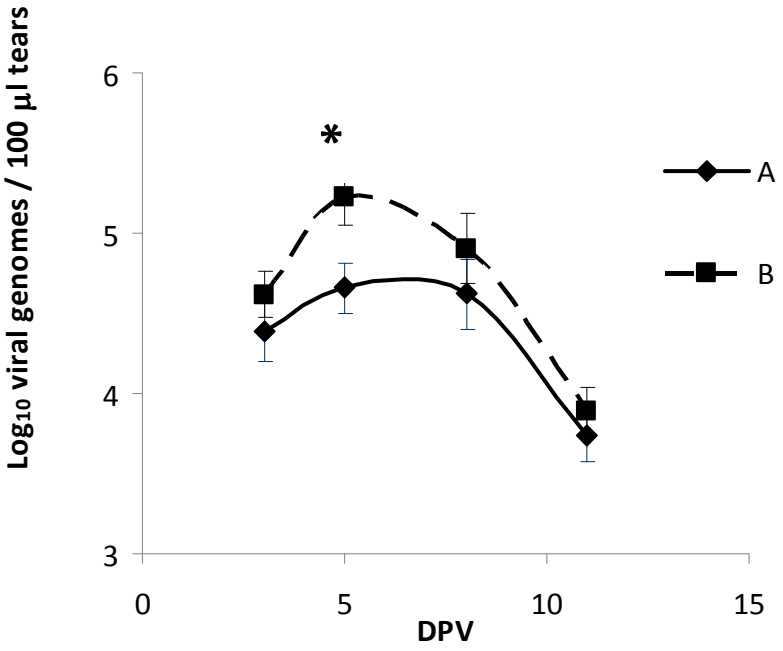

Figure 3. 2: *Viral genome quantification in tears. IBV RNA extracted from tears of chickens vaccinated with different Ark IBV vaccines (n=16) was analyzed by qRT-PCR at the indicated time points after vaccination. Mean log viral genomes were compared between the groups by Student's t test. * indicates values between the two vaccine groups were significantly different. Error bars represent SEM.*

Tracheal and cloacal viral detection

IBV infects and causes pathology primarily in the upper respiratory tract tissues of chickens although nephropathogenic strains and viruses that cause proventriculitis and reproductive tract infection have also been reported. Replication in the gut results in virus shedding through the fecal route. We therefore characterized IBV viral subpopulations present, in tracheal and cloacal swabs in vaccine A and B administered chickens between 5 and 20 DPV.

In the trachea at 5 and 7 DPV, all the chickens vaccinated with either vaccine A or B were positive for viral detection (Table 3.3). However by 20 DPV, only 5 of 16 chickens vaccinated with vaccine A were positive for vaccine viruses in trachea by RT-PCR as compared to 14 of 16 of chickens vaccinated with B vaccine that were still positive, indicating a longer persistence in trachea of vaccine B viruses than vaccine A viruses in vaccinated chickens.

Table 3. 3: *Incidence of tracheal and cloacal viral detection in vaccinated chickens*

Sample source	DPV	Vaccine A		Vaccine B	
Tracheal swabs	5	16/16		15/16	
Tracheal swabs	7	16/16		16/16	
Tracheal swabs	20	5/16	*	14/16	*
Cloacal swabs	7	15/16		11/16	
Cloacal swabs	20	8/16	*	1/16	*

*Incidence statistically significantly different between the two vaccine groups ($P<0.05$)

The presence of Ark IBV vaccine viruses in the cloacal swabs was examined at 7 and 20 DPV. In chickens vaccinated with vaccine A, 15/16 chickens were positive for vaccine virus at 7 DPV while 11/16 chickens vaccinated with vaccine B were positive on this day. When the chickens were examined again at 20 DPV, a significantly higher number of chickens ($P<0.05$), 8/16, vaccinated with vaccine A were still positive for vaccine viruses as compared to only 1/16 in the group that received vaccine B. These results indicate that different Ark IBV vaccine viruses establish themselves differentially in different tissues in vaccinated chickens. While vaccine B viruses tended to be detected for a longer period in the trachea, vaccine A viruses were detected longer in cloacal swabs.

Viral subpopulations detected in trachea

The presence of heterogeneous viral subpopulations with S1 gene polymorphisms may result in selection of certain viral subpopulations in particular tissues if the specific polymorphism allows selective replication advantage in those tissues. We therefore sequenced a portion of the S1 gene of Ark IBV subpopulations detected in trachea and cloacal swabs of chickens vaccinated with either vaccine A or B between 5-20 DPV.

As found in tears, chicken vaccinated with vaccine B had mixed viral subpopulations in the trachea with some chickens harboring up to three different subpopulations at any single sampling day 5-7 DPV (Table 3.4). At 7 DPV, a significantly higher number of tracheal samples ($p<0.05$) from vaccine B chickens contained mixed subpopulations than vaccine A (Table 3.5). Six different viral subpopulations previously described (Table 2.2, chapter 2) and also new variants not previously seen with non-synonymous single nucleotide polymorphism were detected in this vaccine group. These variants were included in the previously described subpopulations that they closely resembled in Table 3.4. One of the subpopulations (C4) was detected in 14/16 of the chickens in combination with other subpopulations at 5 and 7 DPV. Interestingly, at 20 DPV when the last positive samples were seen, C4 was detected in all the positive samples mostly as the only subpopulation except in f samples where it was mixed (Table 3.4). The two chickens that did not have this component in trachea at 5 or 7 DPV were found to have this component at 20 DPV. These results indicate that vaccine virus selection occurs over time in the tissues allowing the virus fit for the specific tissue to persist longer. In our study, the C4 subpopulation of vaccine B seemed to have a better adaptation in the trachea over other vaccine subpopulations (Table 3.4).

Chickens vaccinated with vaccine A were also all positive for vaccine virus in trachea at 5 and 7 DPV. Most of the samples from these chickens at both 5 and 7 DPV had the C1 subpopulation (26/32) but occasionally other subpopulation (C4 and C3) could be seen in some chickens (Table 3.5). In 7/32 samples from 5 and 7 DPV, C4 was detected often in combination with C1 (Table 3.5). By 20 DPV only five of the 16 vaccinated chickens were weakly positive for virus presence in the trachea (Table 3. 3 above). Out of these five, only two samples were successfully sequenced and one contained C4, while the other contained a combination of C1 and C3 together (Table 3.5). Comparing the two vaccines it is possible that the predominant vaccine A subpopulation (C1) although able to replicate in the tracheal tissues is cleared rapidly by the host immune response by 20 DPV. Interestingly even in the group that received vaccine A, C4 was detected in the trachea samples at a higher frequency in chickens at 5 and 7 DPV (5/16) than had been detected in the tears of the same chickens (3/16). This finding may suggest that C4 may have replication advantage in the trachea compared to other vaccine subpopulations.

Table 3. 4: *Viral subpopulations in the trachea of chickens vaccinated with vaccine B 5, 7 and 20 DPV*

Chick #	5 DPV	7 DPV	20 DPV
B1	C1,C4,C5,V	C1,C2	C4
B2	C4,V	C4	C4
B3	C4	C1,C5	C1,C4
B4		C1,C4	C4
B5	C1,C2,C4	C1,C4	C4
B6	C1,C3,C4	C1,C4	C4
B7	C1,C4	C1,C4	C4
B8	C2,C3,V	C1,C3,C4,V	C3,C4
B9	C3,C4	C2,C4	C4
B10	C1,C5	C1,C5,V	
B11	C4,V	C2,C4	Unknown
B12	C4,V	C4,V	C1,C4
B13	C4	C2,C4	
B14	C4	C3,C4	C4
B15	C4	C4,V	C1,C4
B16	C1	C1,C4	C1,C4

Table 3. 5:*Viral subpopulation dynamics in the trachea of chickens vaccinated with vaccine A at 5, 7 and 20 DPV*

Chick #	5 DPV	7 DPV	20 DPV
A1	C1,C4	C1,C4	C4
A2	C1,C4	C1,C4	
A3	C1	C1	
A4	C4	C1,C4	
A5	C1,C3	C1,C3	
A6	C1	C1	
A7	C1	C1	
A8	C1	C1	
A9	C1,C3,C4	C1,C4	C1,C3
A10	C1	C1	
A11	C1,C3	C1,C3	
A12	C1	C1	
A13	C2,C1,C4	C4,C3,C1	
A14	C1,C3	C1,C3	
A15	C1,C3	C1,C3	
A16	C1	C1	

Viral subpopulations in the cloaca

IBV strains replicating in the gut are shed into the environment and can be horizontally transferred to pen-mates through fecal shedding. We therefore examined viral subpopulations in cloacal swabs, in chickens vaccinated with vaccine A or B. The subpopulation structure seen in the cloacal swabs in vaccine A administered chicken was different from those found in the trachea in the same day (Table 3.6). While only 5 chickens from vaccine A group had C4 in the trachea on 7 DPV, 11 birds were found to contain this component either alone or in combination with C1 or C3 on this day in cloaca. However by 20 DPV, C1 was found in four chickens, two of them in combination with either C4 or C3. C3 was detected in two out of the five positive samples while it had

only been detected in one sample at 7 DPV. It is possible that C3 may have been shed to the environment and spread to cage mates. The viral subpopulations in chickens vaccinated with vaccine B was similar to those found in the trachea of these chickens with C4 being present in all except one sample in combination with C1, V, C5 or other variants. Except for a single bird that contained C4 subpopulation at 20 DPV all the other chickens that received vaccine B were negative for virus detection in cloacal swabs by this day.

Table 3. 6: *Viral subpopulation detected in cloacal swabs of chickens vaccinated with two different Ark IBV vaccines*

	Vaccine A			Vaccine B	
Chicken#	7 DPV	20 DPV	Chicken #	7 DPV	20 DPV
1	C1,C4		1	V,C4,C1,C3v	
2	C4		2	V,C4,C1	
3	C1,C4		3	C4	C4
5	C4		4	C1,C4	
6	C1,	C1	5	C4	
7	C4	C1	6	C4	
8	C1	C1,C4	7	V,C4,C5	
9	C1,C4		8	C1,C4,v	
10	C1,C4		9	C4,C5	
11	C1,C4,C3		10	C4	
12	C1,C4		11	C1	
13	C1,C4	C3			
14	C4	C1,C3			
15	C1				

Mucosal antibody induction

Mucosal antibodies are known to play a role in protection against IBV. We compared IgA and IgM antibodies in tears from chickens vaccinated with the two Ark vaccines A and B. Mean log IBV specific IgM ELISA absorbance values for pre-immune tears, 5 and 8 DPV from chickens vaccinated with the two vaccines were not statistically significantly

different (day 0-8, Fig. 3.3A). For both groups, IgM ELISA values increased significantly from day 5 to 8 DPV reaching a peak on 8 DPV. The IgM values then started to drop from this day reaching pre-immune levels by 17 DPV. The drop between 8 and 11 DPV was significantly higher (*P=0.06*) for chickens vaccinated with vaccine A than that of chickens vaccinated with vaccine B. IgM values in group B remained elevated a little longer before dropping compared to group A. Comparing the two vaccine groups, vaccine B group had significantly higher ($p<0.05$) specific IBV IgM at 11 DPV than those vaccinated with vaccine A (Fig. 3.3A).

IgA plays a major role in protection of mucosal surfaces from infectious agents. For both groups of chickens, IBV-specific IgA antibodies increased significantly from pre-immune levels by 11 DPV. IBV-specific IgA antibodies in tears of the chickens vaccinated with vaccine B peaked earlier (11 DPV) than vaccine A (17 DPV) and also were of higher magnitude than those achieved by chickens vaccinated with vaccine A (Fig. 3.3 B). Once these significantly higher levels were achieved in both vaccination groups, they remained high until 21 DPV, the last day of evaluation (Fig. 3.3B).

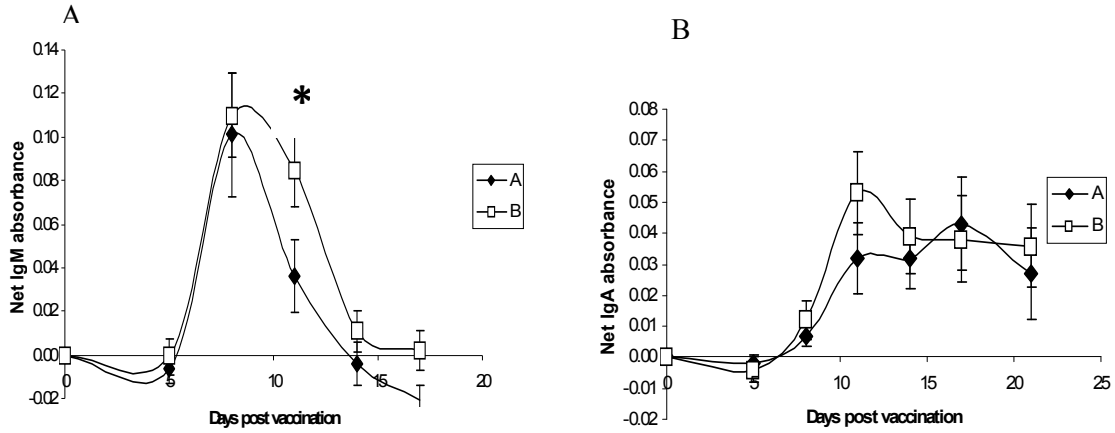

Figure 3. 3: *Mucosal IBV-specific IgA and IgM in chickens vaccinated with two different Ark serotype vaccines. Tears were collected from chickens vaccinated with either vaccine A or vaccine B at the indicated time points. ELISA to detect IBV-specific IgM or IgA antibodies was carried out on tears diluted 1:10 and the absorbance readings normalized to pre-immune values for each chicken by subtracting each pre-immune absorbance value from post vaccination value for each chicken. Net absorbance values were compared between the two groups. * indicates values between the two vaccine groups were significantly different (P<0.05). Error bars indicate SEM.*

Systemic immune response

Systemic IBV-specific antibody levels for both vaccine groups had two peaks, 17 and 28 DPV, the second being higher than the first (Fig. 3.4). In both vaccine groups, IBV-specific antibody values rose sharply from 8 DPV to reach statistically significant higher levels than pre-immune absorbance by 11 DPV (P=0.0007 for vaccine A and P=0.0005 for vaccine B; Fig. 3.4). IBV-specific antibodies for both vaccine groups continued to rise until 17 DPV, reaching the first peak on this day after which the values fell slightly. Vaccine B group had statistically higher serum IBV specific antibodies than vaccine A

group at 21 DPV (P<0.05) and 28 DPV (P=0.05). For vaccine group B, the mean S/P ratio of greater than 0.2 (assay IBV positive cut off value) was achieved by day 11 and remained positive until the last sampling day (28 DPV). For vaccine A group, this value was reached later. The number of IBV seropositive chickens was significantly higher (P<0.05) for vaccine B than A at 21 DPV.

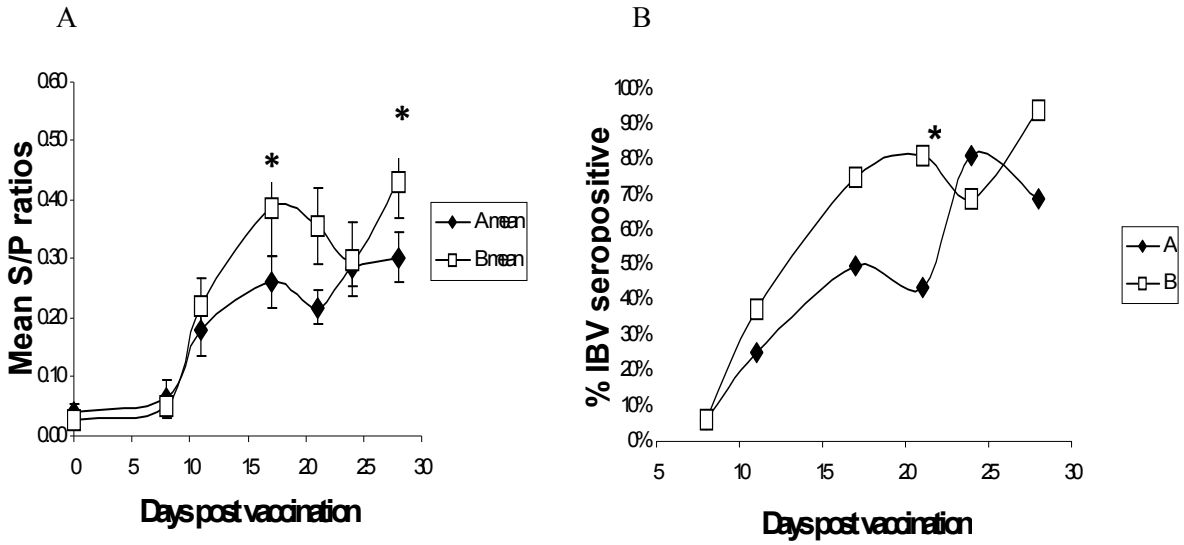

Figure 3. 4: *IBV specific antibodies in sera of chickens vaccinated with two Ark serotype vaccines. A) Sera from chickens vaccinated with either of the vaccines were collected at the indicated time points. IBV-specific ELISA was performed on all samples and the S/P ratio calculated according to the manufacturer's protocol. The mean S/P values were compared between the two groups using the student's t test. * indicates data points that are significantly different (p<0.05 between groups). Error bars indicate SEM. B) The number of IBV seropositive chickens (S/P>0.2) were determined in the two vaccine groups and their incidences calculated for the indicated days. Incidences were compared*

*by Fisher's exact test. * incidences significantly different between vaccine groups on this day (P<0.05)*

Discussion

We previously reported that IBV Ark serotype vaccines, unlike other serotype vaccines, have minor vaccine subpopulations, some of which are selected as early as three days post vaccination in experimentally inoculated chickens. In addition the proportion of selectable subpopulations differed among vaccines (van Santen & Toro, 2008). In general vaccines with a higher proportion of selected subpopulations (A and C) resulted in a higher viral load and stronger systemic immune response than those with a lower amount of selected subpopulations (B and D). We also found one vaccine had no single subpopulation selected but instead had up to six different subpopulations selected in individual chickens and sometimes as mixed subpopulations in individual chickens. In this study we compared two Ark vaccines (A and B) with different proportions of selectable virus and which also resulted in different viral loads, immune response and vaccine reaction. Comparison was made on viral replication levels, tissue preferences and persistence and induction of the mucosal and systemic humoral immune responses in vaccinated chickens. As previously reported (van Santen & Toro, 2008) we found that one of the Ark vaccine (A) resulted in a predominantly single homogenous viral subpopulation in vaccinated chicken while in sharp contrast the other Ark vaccine (B) from a different company resulted in multiple viral subpopulations selected in tears and trachea of vaccinated chickens. We also found that some of the vaccine subpopulations in vaccine group B were rapidly cleared by the host and were not detectable beyond 5 DPV. In particular the major vaccine subpopulation (V) and another minor component (C5)

were not detected at later time points especially in chickens where they were detected earlier in mixed populations. This points to a fitness variability of the various subpopulations resulting in early clearance of some subpopulations due to being outcompeted by higher fitness subpopulations amongst them.

The presence of heterogeneous subpopulations in vaccines is not a unique feature of IBV live attenuated vaccines as it has also been reported in other RNA viruses like infectious bursal disease virus (IBDV) live attenuated vaccines (Jackwood & Sommer, 2002). Live attenuated DNA vaccines like infectious laryngotracheitis virus (ILTV) vaccines derived from passage in embryonated eggs are also reported to harbor multiple viral subpopulations (Garcia & Riblet, 2001). The significance of existence of multiple viral subpopulations in live attenuated vaccines is not fully elucidated. In the event that some of the viral subpopulations are residual virulent subpopulations from the original parent virus, these may be selected when the vaccines are administered in their respective hosts resulting in virulence. This phenomenon has been observed in chicken embryo origin ILTV vaccine, which was back-passaged several times in chickens (Guy *et al*., 1991) and also some IBV vaccines (Hopkins & Yoder, 1984). The presence of multiple genotypes in IBV vaccines has been known for years and some studies suggest that it may be advantageous as it increases antigenic diversity and hence the breadth of protection (Nix *et al*., 2000).

Our previous study detected significantly higher viral loads in chickens vaccinated with vaccine A (with higher proportion of selectable virus) than vaccine B. In addition vaccine B unexpectedly induced a severe vaccine reaction than vaccine A. Surprisingly in this study we detected higher viral loads in the group of chickens that

received vaccine B than A at 5 DPV. However in this study we used a higher dose of the vaccines (1.5 $X10^5$ EID_{50}) than used in the previous study (4 $X10^4$ EID_{50}). This may have resulted in the selected subpopulations being detected in all the chickens vaccinated with vaccine B unlike in the previous study. In addition we found significantly higher number of subpopulations per chicken in the group that received vaccine B than A. It is possible that increased viral heterogeneity in Ark IBV vaccine B vaccinated chickens resulted in enhanced replication ability in eye tissues leading to significantly higher viral load being detected in the group of chickens that received vaccine B than A. This benefit was not achieved until 5 DPV, the time at which peak viral load was detected. In comparison with vaccine A group with single a homogeneous subpopulation selected in chickens, vaccine B group with genetic heterogeneity in the vaccinated chickens achieved significantly higher (10X) viral copy numbers at 5 DPV and thereafter maintained higher viral loads. The reasons behind this enhanced replication ability of heterogeneous subpopulations as compared to homogenous viral subpopulations are not well understood. We suppose however that the genetic heterogeneity in the vaccine (variation is detected within S1 gene which is important for attachment to host receptors) gives the virus ability to infect and replicate in a broad variety of cells found in the eye tissues resulting in higher viral load values. This has been previously suggested as an advantage of genetic heterogeneity in viral populations by allowing the virus to adapt to changes in host environment and also ability to infect different host tissues (Domingo *et al*., 1998; Jackwood *et al*., 2002; Hanson, 1988). In support of enhanced tissue tropism in human viral populations with genetic heterogeneity, Vignuzzi *et al*. (2005) reported that decreasing polio viral diversity by using a poliovirus carrying a high fidelity polymerase resulted in an attenuated

phenotype with loss of neurotropism. When viral diversity was expanded by chemical mutagenesis before infection, neurotropism was restored. Gallardo *et al*. (2010) also found that the different Ark vaccine subpopulations could be detected in eyes, trachea, cecal tonsils, and also in the reproductive tract of vaccinated chickens at varying frequencies indicating an ability of heterogeneous vaccine to infect multiple organs.

Examining the trachea where IBV predominantly causes pathology, we found that several viral subpopulations could be detected at 5 and 7 DPV in mixed infections in individual chickens vaccinated with either of the vaccines. Interestingly when we examined the trachea at 20 DPV in the same chickens, those receiving the vaccine containing a homogenous selectable subpopulation (A) were negative for virus detection while those vaccinated with the vaccine with heterogeneous subpopulations (B) were still positive for vaccine viruses. We found that most of the viral subpopulations detected earlier on in the trachea in chickens vaccinated with vaccine B were no longer present at 20 DPV. Interestingly all the positive tracheal samples had the C4 subpopulation detected mostly as the only subpopulation except in a few samples where it was found as mixed subpopulations. We hypothesize that among the many viral subpopulations detected earlier, only a few are best fit for replication in the trachea, in our case, the C4 subpopulation may have replication advantage in the trachea tissues compared to the other subpopulations. It is possible that the more severe vaccine reaction reported in chapter 2 in group vaccinated chickens may have been due to the presence of this subpopulation. In agreement with our findings, Gallardo *et al*. (2010), studying the spatial evolution of Ark vaccine viruses in vaccinated chickens reported a higher incidence of

subpopulation C4 in tracheal tissues compared to other vaccine viral subpopulations examined.

In cloacae, virus detection was done at 7 and 20 DPV for the two groups of chickens receiving the two different Ark vaccines. The two groups were positive for virus at 7 DPV but at 20 DPV no virus was detected from chickens receiving vaccine B as opposed to vaccine A, which still had 50% positive chickens. At 7 DPV post vaccination most samples from the vaccinated chickens had mixed subpopulations but at 20 DPV, the last day of sampling, mostly two subpopulations were detected C1 and C3. The results of our study point to an advantage in heterogeneity in giving the virus ability to select which subpopulation is best for the tissue over time. While broad infection may be good for the virus for its survival, the full benefit to the host in stimulating broader immunity is still not fully understood and remains to be further explored. In particular persistence of vaccine viruses may result in mutation and recombination of these variants within the host and possible reversion to virulence. Selection of virulent subpopulations through back-passage is also a potential challenge (van Santen & Toro, 2008).

Protection against IBV is from both local and systemic immune responses. Vaccine heterogeneity has been suggested as a probable cause of increased breadth of protection in vaccinated subjects (Nix *et al*., 2000; McKinley *et al*., 2008). While serum antibodies are routinely used to determine flock protection, no correlation to protection has clearly been found (reviewed in Cavanagh, 2007). Some studies have however demonstrated a correlation between IgA in tears and protection (Toro & Fernandez, 1994) and hence antibody levels in tears may be used as indicators of protection. We compared the level of antibody induction by the two different Ark serotype vaccines in

groups of vaccinated chickens in both tears and serum. In tears, we found that vaccine B induced significant increases in IgA from pre-immune values earlier than the vaccine A. IgA peak values were achieved at 11 DPV for vaccine B group of chickens while IgA values in vaccine A group of chickens did not peak until 17 DPV and the peak was smaller. In addition, IgM values at 11 DPV for the B vaccine group were significantly higher than for the vaccine A group. Furthermore total serum antibodies achieved for the higher heterogeneity vaccine were significantly higher at 21 and 28 DPV than those of the vaccine with less heterogeneity. We suppose that the ability of the vaccine with more viral diversity (vaccine B) to infect multiple cell types and tissues in the vaccinated chickens resulting in a higher viral load possibly resulted in the higher IgM 11 DPV, and total antibody induction detected in this group at 21 and 28 DPV. It is also possible that among the many viral subpopulations in this group, some subpopulations may have infected the professional antigen presenting cells resulting in a greater stimulation of immune responses.

The objective in designing live attenuated vaccines is to strike a balance in induction of immune response and ability to cause pathology in the host (Bijlenga *et al.*, 2004). While we find an enhanced immune response with the current heterogeneous vaccine in this study as compared to the homogenous vaccine, effects on the broadness of the protection of the induced immune response and effects of the viral subpopulations on pathology were not analyzed. Furthermore we also find one subpopulation (C4) from the same vaccine (B) being detected in the tracheal tissue longer than desired for vaccine viruses. All these factors would need to be clearly examined in addition to the enhanced immune response by the vaccine to fully understand if any advantage exists in using an

IBV vaccine with higher viral heterogeneity. Further evaluation of these vaccine subpopulations is required preferably in their cloned state so that their characteristics can be more clearly understood.

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CHAPTER 4

TRANSMISSION OF ARK SEROTYPE INFECTIOUS BRONCHITIS VACCINE VIRAL SUBPOPULATIONS TO NON-VACCINATED CONTACT BIRDS

Abstract

IBV Ark-genotype (including ArkDPI, Ark 99, and Ark-like) viruses are the most frequent isolates detected in field samples from avian respiratory cases testing positive for infectious bronchitis virus (IBV) in the southern US. We previously reported that minor viral subpopulations within commercial avian infectious bronchitis Ark serotype vaccines are positively selected in chickens within 3 days of inoculation. The most frequently selected viral subpopulations are more similar to the unattenuated field isolate from which the vaccines were derived than the vaccine strain in their spike (S1) gene. To further explore the significance of these minor vaccine subpopulations we examined the ability of these viruses to be transmitted to contact non-vaccinated birds. We inoculated duplicate groups of five fourteen day-old SPF chickens ocularly with three different doses of: 1.6×10^3, 3.2×10^4, or 3.2×10^5 EID_{50} IBV-Ark vaccine to simulate the field vaccine dose variability that occurs during mass administration. Three days post vaccination (DPV), we introduced five non-vaccinated chickens into each of the isolators with vaccinated chickens. The sequence of a portion of the S1 gene of the infectious bronchitis virus (IBV) present in tears and tracheal samples from both vaccinated and contact birds was determined to identify the viral subpopulation(s) present.

We observed that different vaccine subpopulations were selected in vaccinated chickens depending on the dose of vaccine applied although one subpopulation (C4) was found across all groups. Our results suggest that not all viral subpopulations detected in vaccinated birds are capable of being transmitted to contact non-vaccinated birds. In particular, the major vaccine viral subpopulation detected at high incidence in low-dose vaccinated groups was not detected in any of the contact birds in the same groups. Two specific minor vaccine subpopulations (C4 and C1) were more frequently detected in contact birds than any of the other vaccine subpopulations irrespective of the vaccine dose group. Ability for some vaccine viruses but not others to be transmitted to contact birds may reflect variability in fitness of these viruses to replicate in the upper respiratory tissues of the chicken and/or sensitivity to host's innate immune response. High fitness viral subpopulations may have the ability to persist longer in vaccinated flocks, which may give them time to mutate or recombine with other IBV viruses resulting in new more virulent variants.

Introduction

IB disease is a highly infectious respiratory disease of economic importance in the poultry industry worldwide. The disease may also be accompanied by pathology in kidney, oviduct and the gut for some IBV strains (Cavanagh, 2007; Cavanagh & Gelb, 2008; Ignjatovic *et al*., 2002; Liu & Kong, 2004). The disease is more severe in the young causing variable mortality mainly due to secondary bacterial infections (Matthijs *et al*., 2003; Landman & Feberwee, 2004). In recovered broiler birds, the disease is associated with reduced weight gain and condemned carcasses due to air sacculitis at slaughter. In layers, reduced egg production, low internal quality and eggshell

abnormalities have been reported (Cavanagh, 2003; Cavanagh & Gelb, 2008; Weiss & Navas-Martin, 2005). In natural infection, disease is transmitted from one bird to the other through aerosol route, but ingestion of virus shed through feces and respiratory secretions can also serve as a source of infection (Ignjatovic, 2000). Control of IBV worldwide is mainly by use of live attenuated vaccines although inactivated vaccines are also used in layers and broilers (Cavanagh & Gelb, 2008). Development of live attenuated vaccines involves multiple passages in embryonating chicken eggs whereby the virus becomes adapted to the chicken embryo but less virulent for the original host (chicken). In viruses that exist as quasispecies, adaptation to a new host involves not only selection of variants that are better suited to replicate in the new host but also recombination and/or mutation of these variants (Fang *et al*., 1995). Although horizontal spread of vaccine virus from vaccinated to the non-vaccinated contacts is thought to play a beneficial role in covering birds that were not covered during initial vaccination (Matthijs *et al*., 2008), concern exists over the possibility of reversion of these vaccine viruses to virulence through back passage in the original host. Reversion to virulence of vaccine strains through back passage has already been reported for infectious laryngotracheitis virus (Guy *et al*., 1991), infectious bursal disease virus (Muskett *et al*., 1985) and IBV (Hopkins & Yoder, 1983, 1986). Live attenuated vaccines may contain low copy number residual virulent subpopulations in the vaccine strains, which may be selected once the vaccine is administered back to the original host. Existence of multiple subpopulations has been found in infectious bursal disease virus vaccine and wild type strains (Jackwood & Sommer, 2002), IBV vaccine and wild type viruses (Nix *et al*., 2000), bovine coronaviruses (Zhang *et al*., 2007), murine hepatitis viruses (Adami *et al*.,

1995) and feline coronaviruses (Battilani *et al*., 2003). Selection of virulent subpopulations has been found to occur for mildly pathogenic avian influenza virus (H5N2) (Brugh, 1988), live attenuated turkey rhinotracheitis vaccine (Naylor & Jones, 1994) and infectious laryngotracheitis vaccine (Garcia & Riblet, 2001). In contrast, Nix *et al*., (2000) suggested that existence of viral heterogeneity in IBV vaccines may be beneficial in inducing a broad immune response.

In the US, several IBV serotypes are in circulation, some that are widely distributed and others that are found within particular geographical regions. The most commonly detected strains in the southeastern US are Ark-genotype (including ArkDPI, Ark 99, and Ark-like) viruses (Jackwood *et al*., 2005; Toro *et al*., 2006; Nix *et al*., 2000) while nationally, the Ark-genotype IBV leads followed by Connecticut, Massachusetts, and finally DE072 (Jackwood *et al*., 2005). The factors responsible for high incidence of isolation of IBV Ark-genotype viruses from commercial flocks in the southeastern US despite vaccination remain to be understood (Jackwood *et al*. 2005). There are possible scenarios that may lead to this in vaccinated flocks, namely: inadequate immune induction due to application of low titered vaccines (Toro *et al*., 1997), back passage of vaccine viruses occurring in unprotected birds (rolling reaction) or existence of residual virulent subpopulations within attenuated vaccines that are subsequently selected in vaccinated chickens. Recombination of persisting vaccine strains of different serotypes or with field strains in vaccinated chickens may also lead to evolution of virulent variant strains (Ignjatovic *et al*., 2006). We and others evaluated IBV-Ark vaccines currently used in the US and found that they contain varying degrees of heterogeneity based on S1 gene sequencing (van Santen and Toro, 2008; McKinley *et al*., 2008). We also reported

that different Ark DPI-derived vaccines have up to 5 distinct minor viral subpopulations that are rapidly selected in inoculated chickens within three days post vaccination (van Santen and Toro, 2008; Gallardo *et al*., 2010). The selected subpopulations are apparently more fit than the major attenuated vaccine population to replicate in the upper respiratory tract of chickens. The selected subpopulations designated component (C) 1, 2, 3, 4 and 5 (Gallardo *et al*., 2010) have spike (S) sequences differing from major vaccine population (V) in four to eight amino acids (van Santen and Toro, 2008). The objective of this study was to compare the fitness of these IBV Ark vaccine subpopulations by determining their ability to be transmitted to non-vaccinated contact birds in the same environment.

Materials and Methods

Vaccines

A commercial single entity IBV Ark serotype vaccine previously found to contain multiple viral subpopulations selected in vaccinated chickens was used in this study (Gallardo *et al.*, 2010). The lyophilized vaccine was reconstituted in tryptose broth containing antibiotics and titered in 10-day-old embryonated eggs.

Experimental design

Two sets of 14-day-old specific pathogen free chickens were used in this study. All chickens were housed in Horsfall-type isolation units. The chickens were given water and feed *ad libitum*. One set was the vaccinated made up of six groups of five chickens per group. Each two groups (total of 10 birds) received the same vaccine dose and were thus replicates. Three doses of the vaccine were used to simulate the dose variability that occurs in the field (low, intermediate and high). The doses inoculated were: low dose, $1.6X10^3$ 50% embryo infectious doses $(EID)_{50}$; intermediate, $3.2\ X10^4$ EID_{50}; and high,

3.2 X10^5 EID_{50}. These doses correspond to 2, 40, and 400 vaccine doses as stated on the vaccine vial. Tears were collected 3, 6 and 8 days post-vaccination (DPV) from both eyes of each chicken as previously described (Toro *et al*., 1993). The birds were necropsied at 8 DPV. Tracheal swabs were collected by swabbing the upper two thirds of each trachea with swabs and immediately put in two milliliters of phosphate buffered tryptose broth containing antibiotics. The swabs were kept and transported in ice and stored at -85º C until RNA extraction. Three days after vaccination, 5 age-matched contact non-vaccinated chickens were introduced into each isolator with the final number of chickens per isolator being 10. Tears were collected from one eye of each chicken 6 and 12 days post exposure (DPE). The chickens were necropsied at 12 DPE and tracheal swabs collected from each of these chickens as described above.

RNA isolation

Total RNA from tears and trachea swabs was isolated using the TRI REAGENT® LS - RNA / DNA / PROTEIN ISOLATION REAGENT for LIQUID SAMPLES as per manufacturer's protocol (Molecular Research Center Inc., Cincinnati, Ohio, USA). To each tear sample (30-100 µl) and tracheal sample (250 µl), 5 µl of polyacryl carrier (Molecular Research Center Inc., Cincinnati, Ohio, USA), a compound that helps to precipitate small amounts of RNA, was added. The RNA was resuspended in 50 µl water, and then stored at -85° C until use. 5 µl of undiluted RNA was used for RT-PCR.

Identification of selected viral subpopulations

cDNA to a portion of the S1 coding sequences in IBV in samples collected from each chicken was prepared from 5 µl of RNA from tears or trachea by RT-PCR using the Qiagen OneStep RT PCR kit (Qiagen, Valencia, California, USA). The primers used

were S17F and S18R (Gallardo *et al*., 2010), which amplify a 750-bp product, including the 5' half of the S1 sequence. Products were purified using QIAquick PCR purification protocol (Qiagen, Valencia, California, USA) as per manufacturer's recommendation. The purified products were submitted to Massachusetts General Hospital DNA core facility (Center for Computational and Integrative Biology, Cambridge, Massachusetts, USA) for sequencing using S1R (5'CATCTGAAAAATTGCCAG3') primer. All sequence chromatograms were carefully examined independently by two people to identify nucleotide positions with more than one nucleotide, indicating the presence of more than one subpopulation. For such mixed sequences, proportions of each nucleotide were estimated by measuring the height of the major and minor peaks, in order to estimate the proportions of each subpopulation.

Results

Viral subpopulations in tears of vaccinated chickens

We found the five previously identified viral subpopulations C1, C2, C3, C4, C5 (Gallardo *et al.,* 2010) and the vaccine major population in chickens vaccinated with the IBV Ark serotype vaccine either singly or in mixed subpopulations in tear samples. In addition, subpopulations similar to a previously described subpopulation (C3) but with new single non-synonymous nucleotide polymorphisms in other positions (233, 511, and 593/602) within the sequenced S1 region were also seen. Occasionally, new variants of the other subpopulations including vaccine populations variant (213), C4 variant (355), C1 variant (69) and variant (226) and C5 variant 60 and 69 were detected in some samples. For subsequent analysis the variants were grouped together with the previously described subpopulation to which each was most similar. The frequencies of viral

subpopulations detected in tears differed depending on the dose of the vaccine given. At 3 DPV chickens vaccinated with the lowest dose (1.6 $X10^3$ EID_{50}) had the vaccine major population detected in 10/18 (56%) IBV positive tear samples (Fig. 4.1A). Most of these samples had mixtures of subpopulations. The incidence of this vaccine major population in these groups was significantly higher ($p<0.005$) than the incidence of this population in the intermediate and the high dose groups. By six days after vaccination the number of samples containing the vaccine major population in the low dose groups was reduced significantly ($p<0.05$) to 3/19 (16%). At 8 DPV, only 2/17 (11%) IBV-positive samples in the low dose groups still had the major vaccine population (Fig. 4.1A). The major vaccine subpopulation was detected in the intermediate dose groups in 3/20 (15%) and 2/20 (10%) samples at three and six days respectively and was not detected in the high dose groups at any time. C1 subpopulation was found in 3/18 (17%) IBV-positive samples at 3 DPV in the low dose group, 9/20 (45%) and 9/19 (47%) of the positive samples in intermediate and high dose groups respectively (Fig. 4.1B). The incidence of C1 was significantly higher ($p<0.05$) in the higher dose groups (intermediate and high) than the low dose groups at 3 DPV. Incidence of C1 decreased in all the groups at 8 DPV but not significantly. The C3 and variants subpopulations were found in 4/18 (22%) IBV-positive tear samples at 3 DPV in low dose groups, 6/20 (30%) in intermediate groups, and 4/19 (21%) IBV-positive tear samples in the high dose groups. These subpopulations increased to 8/19 (42%) at six days post vaccination in the low dose groups, while it decreased in both the intermediate and high dose groups (Fig. 4.1C).

C4 subpopulation was detected in 6/18 (33%) IBV-positive tear samples (Fig. 4.1 D), half of them as the only subpopulation and the rest in mixed subpopulations in the low dose

groups at 3 DPV. In the intermediate groups, C4 was detected in 5/20 (25%) samples. In four out of the five samples where C4 was found, it was the only viral subpopulation readily detected in those samples. 15/19 (79%) IBV-positive samples in the high dose group had the C4 subpopulation detected. A slight increase in incidence was detected in the intermediate dose groups at 6 DPV but no change was detected in the high dose groups where the incidence remained high (Fig. 4.1D). At 8 DPV, the C4 subpopulation became the most frequently detected type in both the low 7/17 (41%) and intermediate 9/18 (50%) dose vaccine groups while it remained the most frequent subpopulation in the high dose groups (Fig. 4.1D).

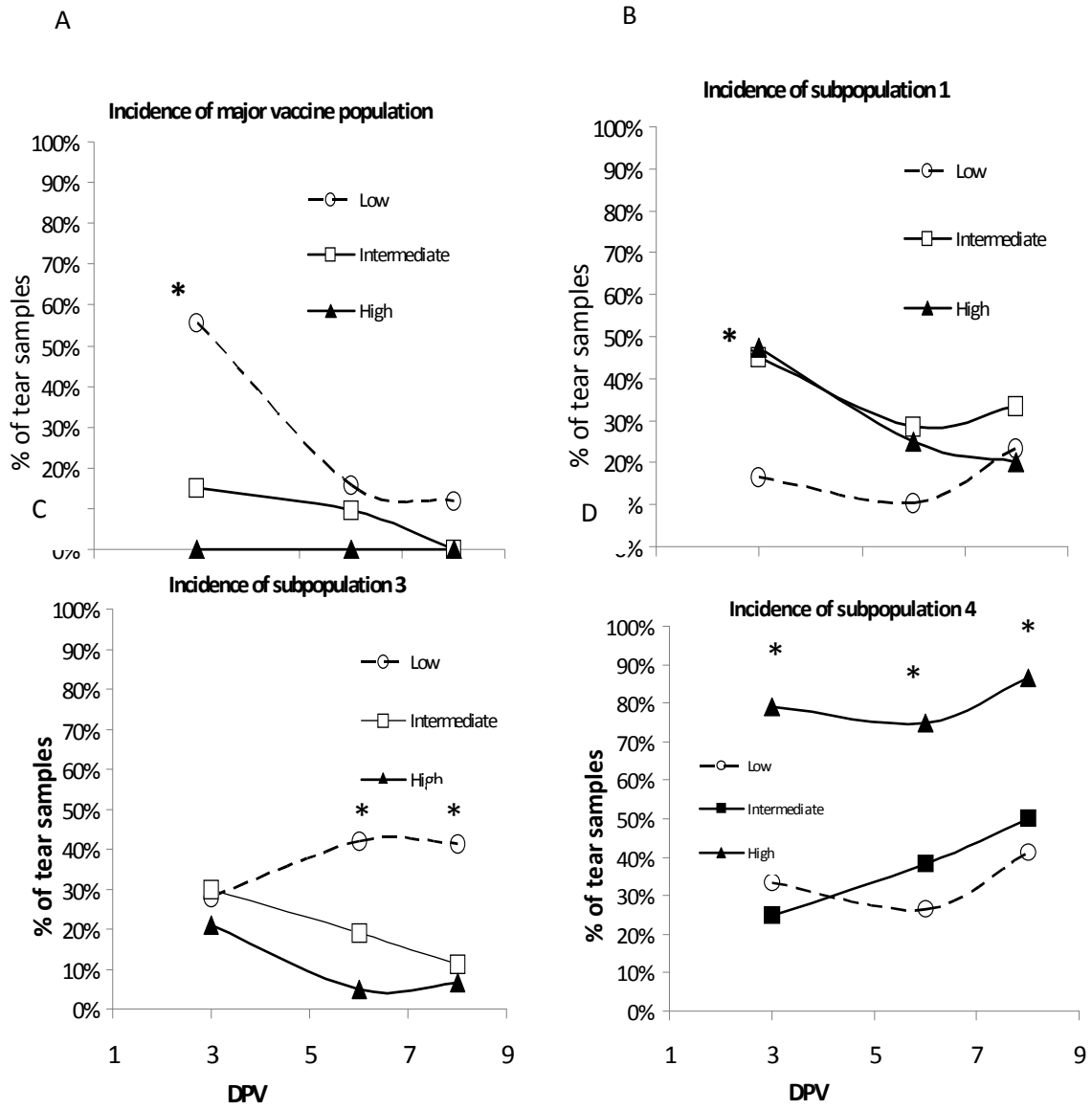

Figure 4. 1: *Viral subpopulations detected in tear samples of IBV Ark serotype vaccinated chickens: Analysis of sequence chromatograms of a section of IBV S1 gene described and used previously to classify the vaccine viral subpopulations was used to determine the presence and incidence of each subpopulation in tear samples of vaccinated chickens. (C) Data points with different letters indicate that incidences in*

those groups were significantly different. (A, B, D) indicates incidences were different from the other two groups.*

Two other subpopulations, C2 and C5 were detected in a few samples at various times (data not shown). C5 was detected mostly in the low dose group while C2 was most frequently detected in the higher dose groups.

Viral subpopulations in trachea of vaccinated chickens

All data regarding subpopulations in the trachea were from tracheal swabs collected at 8 DPV. All subpopulations detected in tears were also detected in trachea of vaccinated birds in the different groups although at different incidences (Fig. 4.2). C4 was the most frequent subpopulation in samples from the low and high dose vaccine groups, 50% and 67% respectively while C1 was the most frequent in the intermediate vaccine groups (67%). C2 was detected in 44% of the tracheal swabs from high dose groups, 22% from the intermediate groups and none in the low dose groups. The C3 and its variant subpopulations were detected only in the low dose groups (30%). The major vaccine subpopulation and the C5 subpopulation were only found rarely in the low dose and high dose groups (Fig. 4.2).

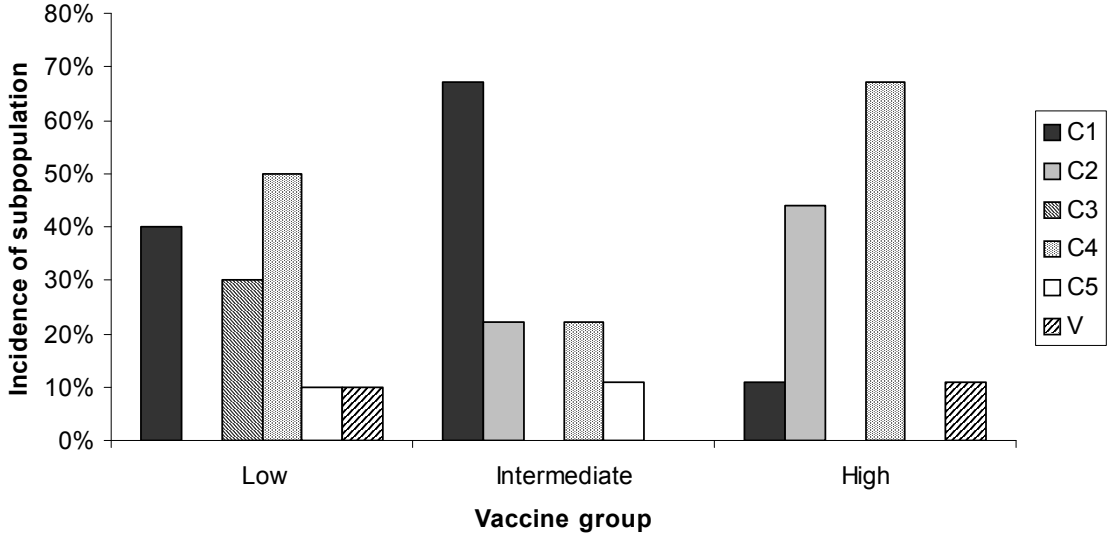

Figure 4. 2: *Viral subpopulations detected in tracheal samples of IBV Ark serotype vaccinated chickens 8 DPV: Tracheal swab samples were processed similarly to tear samples and viral subpopulation incidences determined for each vaccine group.*

Transmission of IBV Ark serotype viral subpopulations to contact birds

Viral subpopulations in non-vaccinated contact birds

Five contact birds per each vaccinated group were introduced into the vaccinated groups three days after vaccination and left together with the vaccinated chickens for five days until the vaccinated chickens were euthanized. All contact chickens in the high dose groups were positive for IBV by 6 DPE, the first day of sampling, while 9 and 7 out of 10 in intermediate and low dose group chickens respectively were positive for IBV at this time. Seventy-seven out of ninety samples (tear and tracheal) from thirty contact exposed birds collected at 6 and 12 days post exposure were successfully amplified and sequenced. In most samples from contact exposed chickens, single subpopulations were detected. The subpopulation detected in tears was in most cases the same one detected in the trachea. In contact group 1, housed with low dose vaccinated group 1, only two subpopulations were transmitted to contact chickens despite having detected seven subpopulations in the vaccinated group. Two out of the five (40%) chickens had the C4 subpopulation while the other three (60%) had the C3 variant 233 subpopulation (Fig. 4.3). It is interesting to note that the C3 variant 233 subpopulation transmitted to these chickens was not the most frequent subpopulation detected in the vaccinated group which this group was contact with. In the contact group 2, which was housed together with low-dose vaccinated group 2, only the C1 subpopulation was detected in the five contact

chickens in both tears and trachea. The major vaccine population, which had been detected in higher incidence than any other subpopulation in vaccinated group 2 at the time the contact birds were added (3 DPV), was not detected in any of the group 2 contact chickens, neither were the six other subpopulations detected in the vaccinated group found in any of the contact chickens (Fig. 4.3). In the contact group 3, housed with intermediate-dose-vaccinated chicken group 3, two subpopulations were detected, C1, 3/5 (60%) and C4, 2/5 (40%) chickens. These two subpopulations were also the most frequently detected subpopulation detected in the vaccinated group 3, which the contact group was housed with. However three other subpopulations (C3, C3 variants and the vaccine major) detected in vaccinated chickens were not detected in any of the samples in the contact group. C4 was the most frequently detected subpopulation in chickens from contact group 4 (3/5, 60%), housed with intermediate-dose vaccinated chicken group 4, despite having found C1, C3, C3 variants and C2 in samples from the vaccinated group 4. A variant of C4 (C4 var 112) not detected in vaccinated chickens in group 4 was found in one contact chicken. C1 and C5 were each detected in one chicken in this contact group. In contact group 5, housed with high dose vaccinated chickens, C4 and C1 variants (69 and 210) were each detected in 3/5 chickens (60%). The C1 variant 210 had not been detected in any of group 5 vaccinated chickens. C2 subpopulation was detected in two samples in this group. Similarly most chickens in group 6 contacts, housed with high dose vaccinated chickens, had the C4 subpopulation (3/5, 60%). A C2 variant 226 was also detected in 2/5 chickens (40%). C1, C1 variant 210, C2 and a C4 variant 433 not detected in the vaccinated groups were each found in one chicken in contact group 6. This data suggests that not all Ark vaccine viral subpopulations detected in vaccinated

chickens are capable of being transmitted to contact non-vaccinated chickens. The finding of some variants not detected in the vaccinated chickens also suggests that spontaneous mutations may have occurred in some of the Ark vaccine subpopulations during replication in the contact chickens.

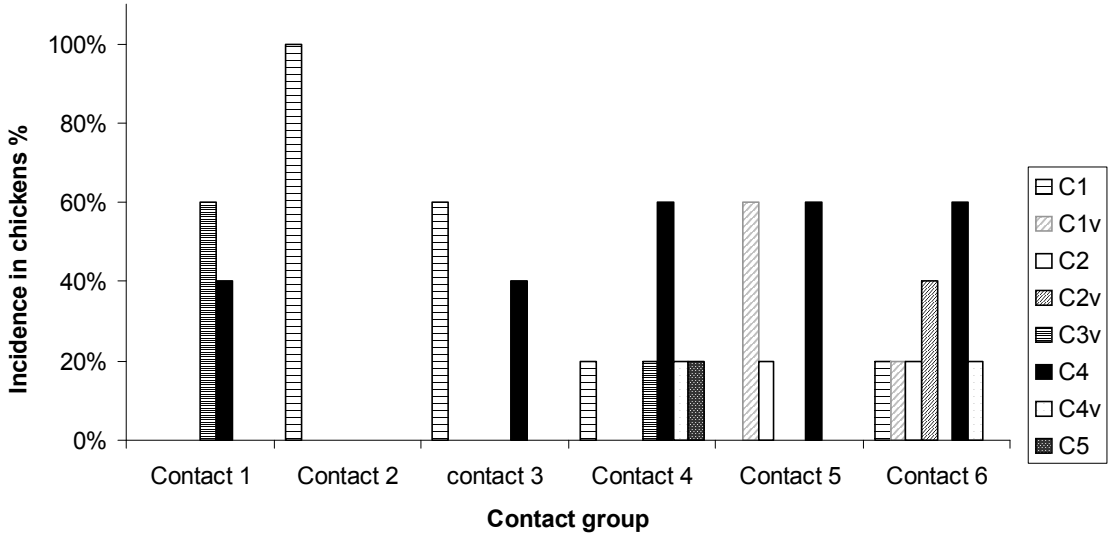

Figure 4. 3: *Viral subpopulations detected in tear samples of contact non-vaccinated chickens. Analysis of sequence chromatograms of a section of IBV S1 gene described and used previously to classify the vaccine viral subpopulations was used to determine the presence and incidence of each subpopulation in tear samples of vaccinated chickens. Contact groups 1 and 2 were housed together with low dose vaccine groups, contact 3 and 4 with intermediate vaccine groups and contact 5 and 6 with high dose vaccine groups.*

Discussion

In this study, we found the six previously described vaccine subpopulations (C1, C2, C3, C4, C5 and V) in tears and tracheal samples of chickens vaccinated with an IBV Ark serotype vaccine. In addition, other variants with new single nucleotide polymorphisms

also resulting in non-synonymous amino acid substitutions in addition to those previously described were found, indicating an even higher viral heterogeneity in this IBV population. The majority of these new variants were more similar to the C3 subpopulation previously described followed by those resembling the C1 subpopulation.

We found that the selected subpopulations in vaccinated chickens depended on dose although the C4 subpopulation was detected across the different dose groups indicating that this subpopulation may be of higher fitness than other viral subpopulations. This subpopulation was the most frequent subpopulation in the high dose group in tear samples throughout the sampling. In the majority of the samples where the C4 subpopulation was detected, it was as a single subpopulation, which further suggests that this subpopulation out-competes the other viral subpopulations. Furthermore, this subpopulation increased in incidence over time in both low and intermediate dose groups to become the most frequent subpopulation at 8 DPV in the intermediate group. In contrast, the major vaccine population detected in high incidence at 3 DPV in the low dose groups was often found in mixed subpopulation. The incidence of detection of the major vaccine subpopulation was also reduced dramatically over time and was only found in a few samples in the low dose groups, and in no samples in either intermediate or high dose groups at 8 DPV. This may suggest that the major vaccine subpopulation replicates poorly in the upper respiratory tract and/or succumbs easily to the chicken's innate immune response while the C4 subpopulation is of relatively better fitness. Although C1 subpopulation was detected in significantly higher number of samples in high and intermediate dose groups at 3 DPV, its incidence decreased by 8 DPV substantially, indicating perhaps its sensitivity to the immune system. The C3

subpopulation and its related variants decreased with time in the intermediate and high dose groups while they increased in the low dose groups. This appears to suggest that this subpopulation may be of better fitness than the major vaccine population but lesser fitness than that of C1 or C4 found in higher incidences in intermediate and high dose groups at 3 DPV.

We found that not all vaccine viral subpopulations found in vaccinated chickens were transmitted to contact chickens. Strikingly the major vaccine population, although detected in high number of samples at 3 DPV in vaccinated chickens that received the low dose, was not detected in any of the contact birds in this group. Instead, minor viral subpopulations detected in fewer samples (C4 and C3 variants) were passed on, suggesting better replication ability and/or higher fitness for these viral subpopulations. The C4 in particular was transmitted to contact chickens in all groups except one of the low-dose groups, irrespective of dose. This subpopulation was detected in tear samples at 6 and 12 DPE and also in the trachea of contact infected birds. This may suggest that this subpopulation, although present as a very minor subpopulation, is of higher fitness than the rest of the subpopulations. The C1 subpopulation was also transmitted to contact birds in the low and intermediate groups, indicating that this subpopulation fitness may also be high relative to other subpopulations except C4. We also found new C3 variants (233, 593/602) and C2 (226) variants not previously detected in vaccinated chickens in our previous studies. Except C3 variant 593/602, the other three variants were also transmitted to contact birds. In addition other variants not detected in vaccinated chickens in this study were also detected in contact birds (C3 variant 511, C4 variant 112, C4 variant 433 and C1 variant 210 and C1 variant 69. It is possible these arose through

spontaneous mutations during replication in the contact birds. Their fitness is difficult to determine since they were found in a low number of chickens. The finding of these variants and the appearance of new ones during a single passage however are significant since they demonstrate the phenomenon of IBV evolution in the host.

Many studies have highlighted the negative consequences of the presence of multiple subpopulations in poultry live attenuated vaccines (Brugh, 1988; Naylor & Jones, 1994; Garcia & Riblet, 2001). Existence of viral subpopulations within live attenuated IBV vaccines has been suggested as a possible source of genetic material for recombination or/and mutation leading to unwanted vaccination consequences (van Santen and Toro, 2008). Recirculation of vaccines viruses is also of concern in large chicken flocks where mass vaccination usually leaves a certain fraction of the chicken population uncovered, which subsequently serves as hosts for vaccine virus rolling reaction (McKinley *et al*., 2008). Ability to passed on to other in contact chickens may give viruses ample time to mutate. This amounts to back passage, a phenomenon already known to result in increased virulence of vaccine viruses. Our findings that some specific vaccine subpopulations within an IBV Ark serotype vaccine are successfully transmitted to non-vaccinated contact birds may imply that these subpopulations have a higher fitness than the rest of the subpopulations. Furthermore the S1 gene sequence of some of these subpopulations is more similar to the unattenuated ArkDPI strain from which they were derived than the vaccine strain (van Santen & Toro, 2008). Whether these high fitness vaccine subpopulations contribute to the high frequency of isolation of Ark-genotype viruses from IBV field cases is not known. Although these subpopulations have not fully been characterized, their ability to be selected and passed on to contact birds is significant

since it means they have potential to re-circulate within the flocks, a factor required for increased virulence of vaccine viruses. These subpopulations therefore warrant more study in their cloned state to determine their ability to induce an immune response, and/or contribute pathology in chicken respiratory system. Evaluating their genetic stability as they are passaged from one chicken to another and ability to persist in the host may also offer important information for IBV vaccine development.

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CHAPTER 5

REPLICATION AND SELECTION OF IBV ARKANSAS VACCINE VIRUSES IN THE PRESENCE OF IBV MASSACHUSETTES SEROTYPE VACCINE

Abstract

Most of IBV field isolates have been shown to be ArkDPI genotype viruses and ArkDPI vaccine strain viruses have also been found to persist longer in vaccinated chickens than other IBV serotype viruses. In addition Ark vaccines protected poorly compared to other IBV serotype vaccines. Experimental data indicate that ArkDPI vaccines are heterogeneous and only minor subpopulations within Ark vaccines replicate efficiently in vaccinated chickens. These Ark vaccine characteristics may result in the inability of the Ark vaccine viruses to compete with other IBV serotypes during multiple serotype vaccine administration. However increasing the Ark vaccine dose administered may increase the amount of high fitness Ark viruses to compete with other serotypes during initial replication. In this study using a quantitative reverse transcription polymerase chain reaction (qRT-PCR), no Ark vaccine virus was found at 3, 5, and 7 DPV in lachrymal fluid of chickens vaccinated with a combined Ark/Mass vaccine irrespective of the Ark vaccine titer used. Ark vaccine virus was only barely detectable at 10 DPV. In contrast Ark vaccine virus replicated well in chickens vaccinated with Ark vaccine alone. Mass vaccine virus on the other hand replicated to relatively high genome copy numbers in all groups and no effect on Mass replication was seen even after increasing Ark

vaccine titers. In the trachea, some Ark vaccine viral subpopulations were able to overcome Mass suppression especially in the group with the highest Ark dose administered. Failure of vaccine viruses to replicate may result in suboptimal immune response and also failure to clear vaccine viruses. Late replication may also lead to persistence of vaccine virus in vaccinated chickens.

Introduction

Avian infectious bronchitis is a highly infectious respiratory disease caused by infectious bronchitis virus (IBV) that continues to be of economic importance worldwide (Cavanagh, 2007). The disease is associated with high economic losses in broilers arising from high mortalities in young chickens due to secondary bacterial infections, reduced and uneven weight gain and condemnation of carcasses at slaughter. In laying birds, losses occur due to reduced egg production, poor egg quality and condemnation of eggs due to eggshell abnormalities (Cavanagh, 2005**)**. Another economic effect of this disease is the cost of control through vaccination where more than two vaccinations for broilers, layers and breeders are required. Rapid appearance of new IBV serotypes that poorly cross protect occurs in the field, requiring constant surveillance and multiple serotype vaccine administration. Appearance of new serotypes and variants is thought to be through spontaneous mutations and/or recombination (Cavanagh *et al*., 1992; Jia *et al*., 1995; Cavanagh, 2005; Bijlenga *et al*., 2004). Protection from IBV disease is both from local and systemic antibodies induced after vaccination or infection (Cook *et al*., 1992; Davelaar *et al*., 1982; Dhinakar Raj & Jones, 1996; Toro & Fernandez, 1994; Toro *et al*., 2006; Nakamura *et al*., 1991; Kotani *et al*., 2000). The important role of cytotoxic T cells has also been documented (Collisson *et al*., 2000; Seo *et al*., 2001).

In the United States (U.S.), the IBV vaccine serotypes most commonly used are Massachusetts (Mass), Arkansas (Ark), Connecticut (Conn) and Delaware (DE072). In most cases due to the existence of multiple serotypes in one region, several IBV vaccine serotypes are combined together during administration for convenience and increasing the breadth of protection (Jackwood *et al*., 2009). In the southeastern U.S., Ark-genotype (including ArkDPI, Ark 99, and Ark-like) IBV continues to be the most frequently isolated IBV associated with respiratory diseases (Jackwood *et al*., 2005; Toro *et al*., 2006), despite intensive use of Ark serotype IBV vaccines in this region for over 20 years now**.** The reasons behind this observation are not clearly understood. Jackwood *et al*., (2009) recently reported poor protection (37.5%-62.5%) against Ark-serotype IBV challenge, compared to 100% protection against DE072 serotype challenge, in commercial chickens following simultaneous administration of the Ark and DE072 IBV serotype vaccines. We and others have recently found that currently-used Ark serotype vaccines differ in their spike (S) gene sequences before and after administration to specific pathogen free (SPF; lacking IBV and antibodies to IBV) chickens, indicating that different minor viral subpopulations contained in IBV-Ark vaccines are rapidly selected in chickens. In contrast, for other IBV serotype vaccines, the viruses re-isolated from the chickens were the same as those originally detected in vaccine vials (McKinley *et al*., 2008; van Santen & Toro, 2008). The minor subpopulations selected from Ark-type vaccines are apparently more fit to replicate in the upper respiratory tract of chickens than the major vaccine subpopulations that are negatively selected soon after vaccination. The proportion of selectable subpopulations within different commercial Ark-type vaccines was found to differ (van Santen & Toro, 2008). The presence of competing and possibly

more fit viruses of other serotypes during multiple serotype vaccination may interfere with selection and replication of these minor Ark vaccine viruses in vaccinated chickens. An earlier study comparing Mass and Ark serotype vaccine virus persistence in experimentally vaccinated chickens found that after simultaneous administration of Mass and Ark vaccines, Mass vaccine was consistently detected in trachea and cecal tonsils early on after inoculation (up to 14 days) but no Ark vaccines were detected at these time points. Instead the Ark vaccines were more consistently detected at later times (21 and 28 days) (Alvarado *et al*., 2006). Persistence of Ark vaccine viruses after multiple serotype administration was confirmed in a recent field study (Jackwood *et al*., 2009).

An important factor that may affect the outcome of vaccination, especially when multiple serotype vaccines are administered simultaneously, is vaccine interference (Vidor, 2007). Administration of multiple agent vaccines is a well-established practice in human and veterinary medicine (Gagic *et al*., 1999 and references therein) and may provide protection against several agents simultaneously, saving time and costs of several vaccinations. This is especially important where multiple serotypes of a disease agent exist in a region and repeated vaccinations are required for these agents. It is also helpful when large numbers of subjects are involved, as in the poultry industry. Chickens in particular have been experimentally vaccinated in ovo with mixtures of vaccine viruses without any vaccine interference (Wakenell & Sharma, 1986; Gagic *et al.,* 1999; Breedlove *et al*., 2011). Simultaneous vaccination with multiple agents however has many concerns and shortcomings. Certain vaccines that are protective when given singly have been associated with poor immune induction when given together with other vaccines, raising the issue of vaccine interference (Gagic *et al*., 1999 and references

therein; Vidor, 2007). This phenomenon has been reported in humans when combined vaccines of varicella, measles, mumps and rubella are co-administered whereby the DNA viruses (varicella) interfered with the RNA viruses (measles, mumps and rubella) replication and hence immune induction (reviewed in Gagic *et al*., 1999). Similar findings were reported by Kanesa-Thasan *et al.,* (2001) with Dengue fever vaccines where no significant levels of viremia or antibodies against serotypes 1, 2 and 4 were induced when the vaccine was administered as a tetravalent vaccine (containing serotypes 1, 2, 3 and 4) but good immune induction was detected when the vaccines were administered as monovalent vaccine in human subjects. In this study, after tetravalent vaccine administration viremia detected and neutralizing antibodies were predominantly for serotype 3. In a similar study but using a monkey model, decreasing the dose of the dominant serotype enhanced immunity to the other serotypes (Guy *et al*., 2009). Interference has also been reported for type A influenza H1N1 and H3N2 live attenuated vaccines (Gruber *et al*., 1996). In this study seroconversion to H1N1 was significantly less frequent in bivalent cold adapted vaccine recipients than in monovalent H1N1 recipients, but seroconversion to H3N2 was not affected**.** Vaccine interference was also demonstrated experimentally for tissue culture origin (TCO) infectious laryngotracheitis virus (ILTV) by monovalent Newcastle disease virus (NDV) (B1) and multivalent (B1, IBV Mass/Ark) vaccines. Both monovalent NDV and multivalent vaccines reduced the replication of TCO ILTV and also protection induced by this vaccine indicated by more pronounced clinical signs following ILTV challenge when the vaccines were given in combination than when the TCO was given alone (Vagnozzi *et al*., 2010). IBV has also been reported to negatively interfere with NDV replication when the two vaccines are

given simultaneously (Gelb *et al*., 2007). Interference of immune induction between IBV serotypes was reported as early as 1968 where the more virulent Mass-41 strain interfered with the milder Connecticut strain neutralizing antibody induction when the vaccines were given simultaneously but not vice versa (Winterfield, 1968). The mechanisms of vaccine interference are not well understood, but they are often described as complex and multifactorial, differing in different vaccine combinations. For example when administered vaccine viruses replicate in the same tissues, competition of the viruses for cells and cell receptors may result in a decrease of immune response to one or more of the inoculated agents (Vidor, 2007). The objective in developing live attenuated vaccines is that vaccine viruses are able to replicate in the relevant host tissues to levels sufficient to produce a protective immune response without causing pathology (Bijlenga *et al*., 2004). Hence the presence of good proportion of Ark vaccine viruses that are able to replicate well in the upper respiratory tissues might be beneficial for Ark IBV protection.

During water or spray vaccine administration in poultry flocks with thousands of birds, it is difficult to have uniform vaccine coverage of the chickens (McMullin, 1985; Fulton *et al*., 2000; Jackwood *et al*., 2009; Butcher & Miles, 1994). For optimum local and systemic immune induction to occur in vaccinated chicken flocks, vaccine viruses have to be available to the chicken in doses that are able to replicate to desired levels (Beard & Brugh, 1975**).** The amount of IBV vaccine viral titer each chicken receives has been shown to affect the humoral response in experimental studies (Toro *et al*., 1997). Higher doses (10^6 EID_{50}) induced both systemic and local antibodies while no detectable antibodies were found with the lowest dose (10^2 EID_{50}). Field vaccinations are further complicated by dilution of vaccines by some producers for economic reasons, which

results in further vaccine dose variability (Jackwood *et al*., 2005)**.** The finding that only minor viral subpopulations within Ark vaccine are fit to efficiently replicate in upper respiratory tract of chicken coupled with multiple serotype vaccine administration in poultry flocks may interfere with IBV Ark serotype vaccination outcome. The objective of this study was to evaluate the possible interference of replication of Ark vaccine virus by IBV Mass serotype vaccine and also the effect of combined administration on selection of Ark vaccine viruses**.**

Materials and Methods

Experimental design

One hundred, two-day-old specific pathogen free chickens were wing-banded and divided into four groups of 25 chickens. The first group received Ark serotype vaccine alone (Ark) containing $6X10^4$ 50% embryo infectious doses (EID_{50}) of Ark vaccine. The other three groups received combined Ark and Mass serotype vaccines at increasing EID_{50} of Ark vaccine but constant Mass vaccine ($1.55X10^5$ EID_{50}). The Mass titer was equivalent to a single dose of Mass serotype vaccine. The first combined group received Ark and Mass vaccine combined in equal doses (single dose based on vaccine vial instructions); this was designated Ark/Mass low dose group (AML). The second group received a single dose of Mass combined with Ark vaccine ($6X10^4EID_{50}$) similar to Ark alone group, and was designated Ark/Mass intermediate dose (AMI). In the fourth group [Ark/Mass high dose (AMH)], Mass and Ark vaccines were given in equal EID_{50} based on the EID_{50} of a single dose of Mass vaccine. The vaccine was administered by eye drop divided into both eyes at two days of age. At 3, 5, 7 and 10 days post-vaccination (DPV),

tears and 5 and 10 DPV tracheal swabs were collected from all birds from each group for testing for individual serotype detection and viral load by qRT-PCR.

The sequence of a portion of the S gene of Ark-IBV present in each tracheal sample from each chicken in each of groups 1-4 at 5 and 10 DPV for Ark and AMI groups was determined to identify which Ark vaccine viral subpopulation(s) was present.

Vaccination

Two single entity commercial IBV vaccines, Massachusetts (Mass) and Arkansas (Ark) were obtained from vaccine companies. The Ark serotype vaccine chosen for this study is one from which we have demonstrated establishment in chickens of at least five distinct subpopulations, as well as dependence of selection on dose of vaccine (Gallardo *et al*., 2010; Chapters 2, 3 & 4, this dissertation). To confirm the dose and EID_{50} of each vaccine being administered, the vaccines were titered in specific pathogen free (SPF) chicken embryos at 9-10 days of embryonation and the titers (EID_{50}) calculated using Reed and Muench method as described (Villegas, 2008). All vaccines were diluted in tryptose broth containing antibiotics. The combined vaccines were obtained by mixing the diluted vaccines in calculated volumes to achieve the desired Ark vaccine/Mass vaccine titer ratios. The chickens were housed in Horsfall-type isolators with chickens receiving same doses and combinations housed together. Feed and water were supplied to the chickens *ad libitum*.

Tear Collection

Tear flow was induced by placing a salt crystal into the eye and tears collected as previously described (Toro *et al*., 1993).

RNA isolation

Viral RNA was isolated from 100 µl of tears and 250 µl tracheal swabs using TriReagent LS (Molecular Research Center., Cincinnati, Ohio,USA) following the directions supplied by the manufacturer, resuspended in 45 µl volume of DEPC-treated water and stored at -85° C until use.

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

A fluorescence resonance energy transfer (FRET)-quantitative RT-PCR based on a portion of the IBV nucleocapsid (N) was designed. Oligonucleotide primers were able to amplify both Ark and Mass vaccine nucleocapsid gene: IBVN 4F 5'AAGAAAACCAGTCCCAGATGCTTG3' and previously designed IBVN 2R 5'GATTCAGAGGAATGAAGTCCCAACG3' (van Ginkel *et al*., 2008). The product size amplified was 236 bp. Probes specific for Ark and Mass vaccine viruses were used. Previously described labeled donor probe and acceptor probe could bind to the Ark vaccine N gene (van Ginkel *et al*., 2008) and were used to quantify Ark genome copies. The 5'-labeled donor probe previously described could also bind the Mass vaccine N gene but the acceptor probe could not. We designed a Mass vaccine specific acceptor probe (5'ACCCTTACCAGCAACCCACACTAT/6-FAM3'). Reactions were performed in 20 µl reaction volumes in a Light Cycler® (Roche) using RNA extracted from either trachea or tears. The reaction was optimized using RNA prepared from the vaccines. Reaction conditions were: reverse transcription at 50°C for 20 min; 95°C for 15 min; 39 cycles of 95°C for 0 sec, 65°C (used during first five cycles), 63°C (used during next six cycles), 61°C (used during next three cycles), or 57°C (used during the last 25 cycles) for 30 sec and 72°C for 30 sec extension for all cycles. During the last 25 cycles, two additional steps of 57°C for 8 sec followed by 62°C for 8 sec were inserted between the

95°C and the 57°C annealing steps to allow vaccine specific probes to anneal, and melt heterologous probes respectively. Fluorescent acquisition was done following the 62° step. Assays were performed using 5 µl RNA prepared from tears or tracheal swabs of each chicken as template. After the amplification program, melting point analysis was used to confirm the specificity of positive reactions. Both Mass and Ark vaccine DNA (10^5,10^4, 10^3, 10^2, and 10 copies of RT-PCR product) standards as well as negative controls containing only salmon sperm DNA were included in each set of reactions. Relative numbers of IBV genomes were calculated based on the standard curve, converted to log values and compared between groups by ANOVA.

For detection of Mass and Ark IBV vaccine in tears and tracheal swabs, melting peak analysis was performed to visualize the presence of two peaks in each of the samples containing both Mass and Ark Vaccine virus and single peaks for samples containing just one of the vaccine viruses.

RT-PCR for sequencing

For generation of a portion of IBV S1 gene cDNA for sequencing to determine which Ark vaccine subpopulation(s) were present, a highly specific and sensitive RT-PCR was used. The Ark specific primers used in this reaction can specifically amplify Ark S gene sequences from a mixture of Ark and Mass IBV RNA containing up to 100-fold excess Mass IBV RNA (unpublished results). The reaction was prepared using Qiagen One-step RT-PCR kit in a 50 µl reaction volume and 5 µl RNA isolated from tracheal swabs was used as template. To identify positive reactions, amplified products were stained using gel green DNA staining dye following agarose gel electrophoresis and visualized under fluorescent light.

Sequencing

RT-PCR products amplified above were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia, California, USA), and sequences were determined by the Massachusetts General Hospital Sequencing Core using the S1R primer (van Santen & Toro, 2008). The chromatograms were examined independently by two people to verify IBV subpopulations found in the samples.

Results

Ark vaccine virus in lachrymal fluid of chickens vaccinated with Ark alone

We assayed for the presence of Ark vaccine virus in lachrymal fluid at 3, 5, 7 and 10 DPV in chickens vaccinated with Ark vaccine alone by quantitative RT-PCR and melting curve analysis. The presence of a single melting peak at 62° C in the test sample similar to that found in the positive standard samples was indicative of the presence of Ark IBV virus in the sample. We found that all the Ark-alone vaccinated chickens were positive for a single clear melting peak at 62°C indicating the presence of the virus (Fig. 5.1). These peaks were detected in samples from all chickens on all the days sampled indicating that Ark vaccine virus was present throughout our sampling period.

We also quantified the amount Ark virus present in these chickens for each of the days sampled. We found that the Ark vaccine viruses replicated to highest levels by 3 DPV and gradually decreased over time. By 10 DPV, the last day of our sampling, the viral genomes had decreased significantly ($P<0.0001$) by about 20 fold (Fig. 5.2).

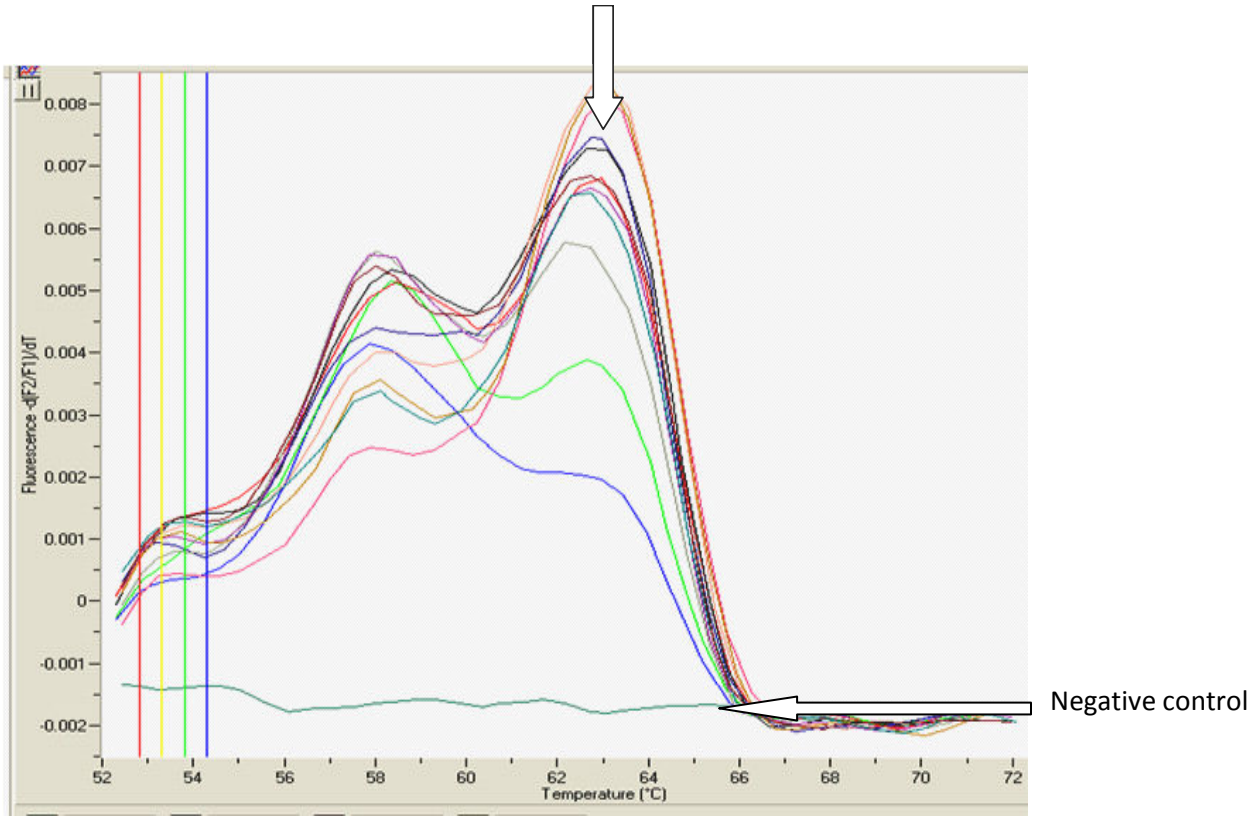

Figure 5. 1: *Representative melting curves in samples positive for Ark virus in chickens vaccinated with Ark vaccine alone. (Top arrow indicates the expected peak in Ark positive chickens). RNA extracted from tears of chickens vaccinated with Ark serotype vaccine alone was assayed using qRT-PCR containing Ark specific fluorescent probes. At the end of the reaction, melting curve analysis was done to determine the specificity of reaction.*

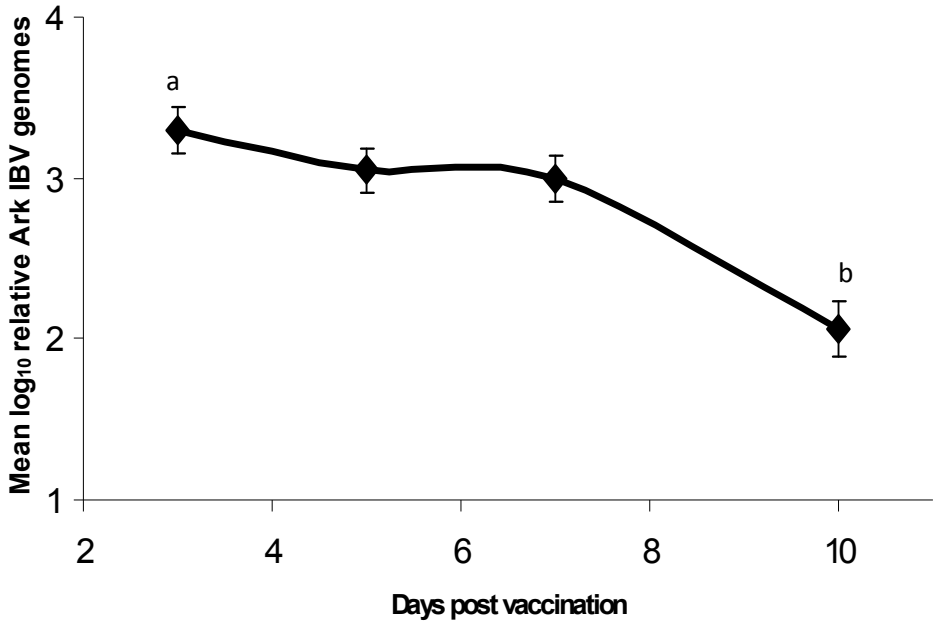

Figure 5. 2: *Relative Ark IBV viral genome levels as detected by Ark specific qRT-PCR in lachrymal fluid of chickens that received Ark vaccine alone, 3, 5, 7 and 10 DPV. Error bars indicate standard error of the mean (SEM). Data points with different letters are significantly different (P<0.05)*

Detection and quantification of Ark vaccine virus in lachrymal fluid of Ark/Mass combined groups

We used quantitative RT-PCR data for detection and quantification of Ark vaccine virus in chickens that received combined Ark and Mass serotype vaccines. Interestingly we found that almost all the chickens in the Ark/Mass combined groups had no quantifiable Ark virus by this assay in the lachrymal fluid through 7 DPV. To confirm the quantification results we further examined the melting curves for peaks at the expected Ark melting peak and found that samples from most chickens had no peak at this region or if present, it was very small indicating that replication of Ark vaccine virus was suppressed in these chickens (Fig. 5.3). The number of samples per group with a peak at

62°C ranged between one and two out of 15 samples analyzed (Fig. 5. 4) but these too had very little, unquantifiable virus. The absence of any detectable Ark vaccine in most chickens was seen through 7 DPV across the three groups of combined Ark/Mass irrespective of the dose of Ark vaccine inoculated. This is in contrast to the group that received Ark vaccine alone, which had Ark vaccine virus detected in all chickens in amounts over 3000-fold our limit of detection during the same sampling period (Fig. 5.2 above).

At 10 DPV however, we started to detect Ark vaccine virus in the groups that received the Ark and Mass combined vaccine, albeit in very low copy numbers. The viral copy number was similar in all the Ark/Mass vaccine groups irrespective of the amount of Ark in the combination. The Ark genome copy numbers at this time point were not different from those detected in the group that received Ark alone (Fig. 5.5).

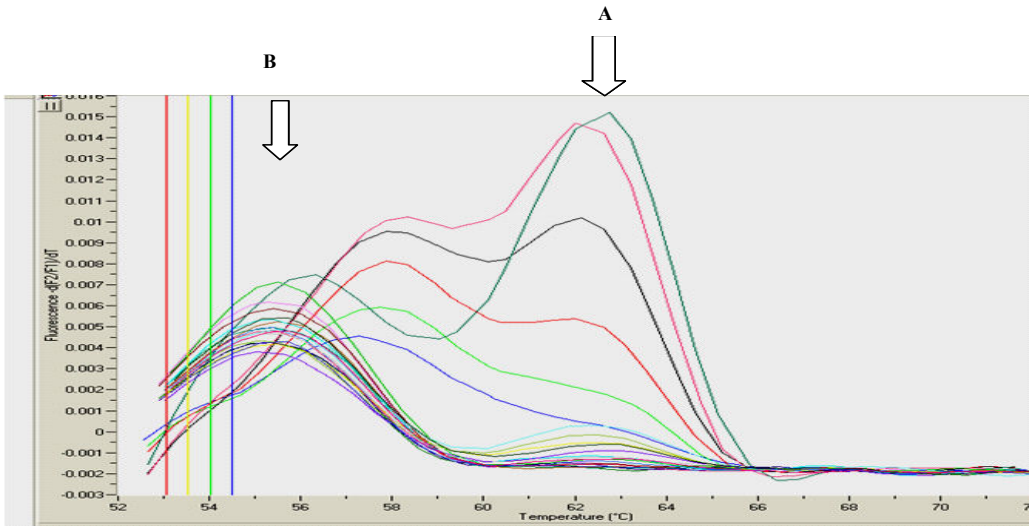

Figure 5. 3: *Representative melting curves from qRT-PCR of Ark/Mass combined groups lachrymal fluid samples 3 DPV showing lack of/or a very small peak where Ark virus peak is expected 62°C (arrow A) but high peaks in the region where Mass peak is*

detected 56°C (arrow B). High peaks at arrow A correspond to the Ark DNA standards peaks (10^1-10^6)

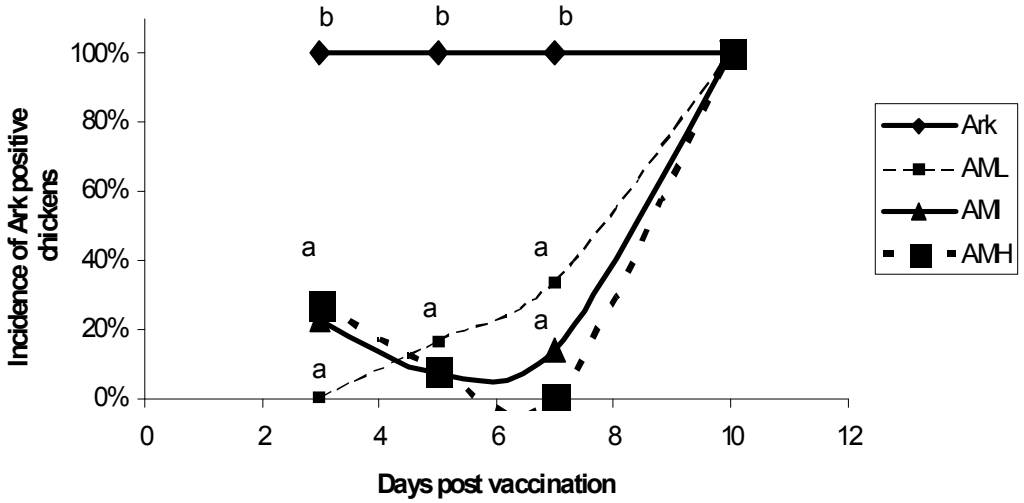

Figure 5. 4: *Incidence of Ark positive chickens in lachrymal fluid of chickens vaccinated with Ark alone or Ark/Mass combined groups (AML, AMI and AMH), 3, 5, 7 and 10 DPV as determined by Ark specific qRT-PCR. Data points marked with different letters are significantly different by Fisher's exact test.*

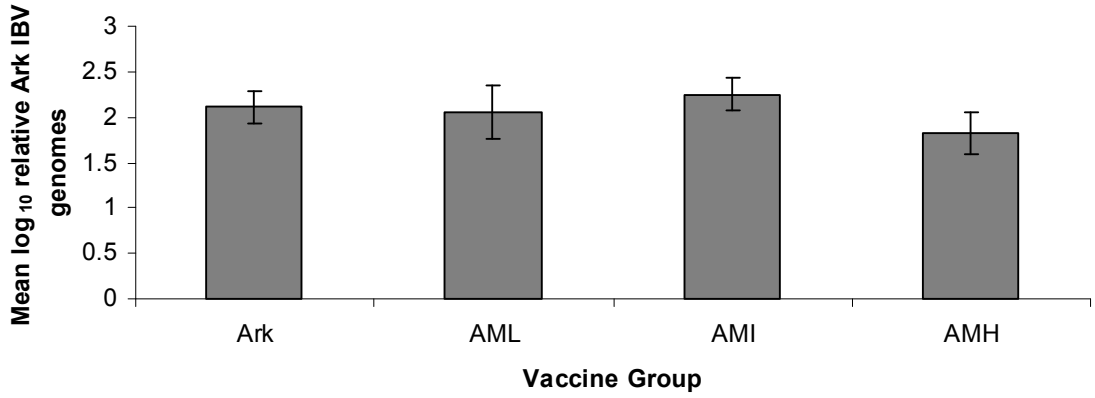

Figure 5. 5: *Relative Ark IBV genomes 10 DPV in RNA samples extracted from lachrymal fluid of chickens vaccinated with either Ark vaccine alone (Ark), or Ark/Mass*

vaccines combined at increasing doses of Ark (AML, AMI, and AMI). Error bars indicate SEM.

Replication of Ark vaccine virus in trachea

Unlike the results seen in the lachrymal fluid, we detected Ark vaccine virus in tracheal swabs in all vaccinated groups at both 5 and 10 DPV, including the groups that received Ark and Mass vaccines combined. However Mass vaccine interference with replication of Ark vaccine virus at 5 DPV was still evident based on the differences detected in the Ark genome copy numbers. The group that received Ark vaccine alone had significantly higher viral genome copy numbers ($P<0.005$) than the two groups that received the low and intermediate doses of the Ark vaccine combined with Mass (Fig. 5.6). The interference of Ark replication by Mass was more pronounced in the group that received Ark at lowest dose where the number of IBV positive was less than the other combined groups (data not shown). Although Mass interference with Ark vaccine replication was evident in the group that received the Ark/Mass combined in equal EID_{50}, the Ark genome copy numbers in this group were not significantly different from the group that received Ark vaccine alone indicating that addition of relatively high dose of Ark vaccine (equal to 250 doses of Ark vaccine) but equal EID_{50} with Mass vaccine might partially overcome the suppression by Mass vaccine. At 10 DPV, all the vaccine groups had similar but lower amounts of Ark virus in the trachea than that detected at 5 DPV (Fig. 5.7).

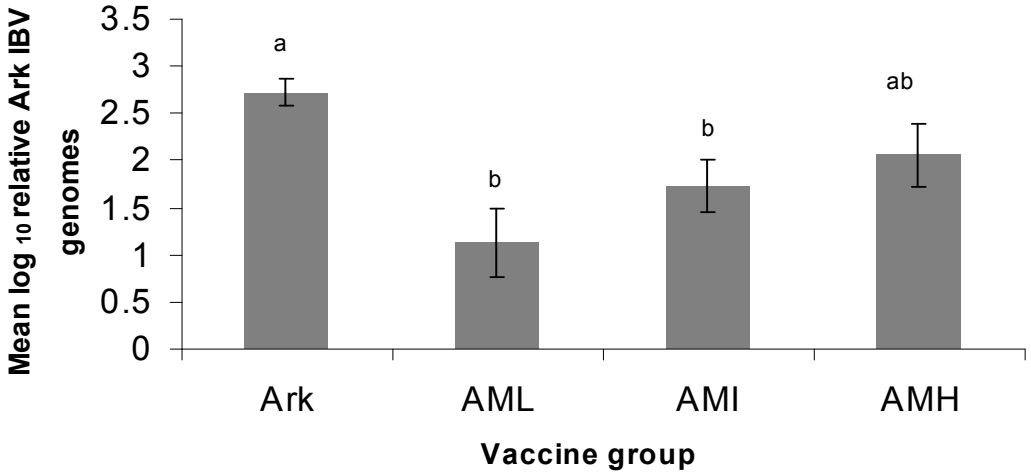

Figure 5. 6: *Relative Ark IBV genomes 5 DPV in RNA samples extracted from trachea of chickens vaccinated with either Ark vaccine alone (Ark), or Ark/Mass combined at increasing dose of Ark (AML, AMI, and AMH). Data points represent mean log relative genomes (n=24). Error bars indicate SEM. Data was analyzed statistically using ANOVA and Tukey's post hoc analysis.* ^{a,b,ab}*Data points marked with different letters are significantly different (P<0.05).*

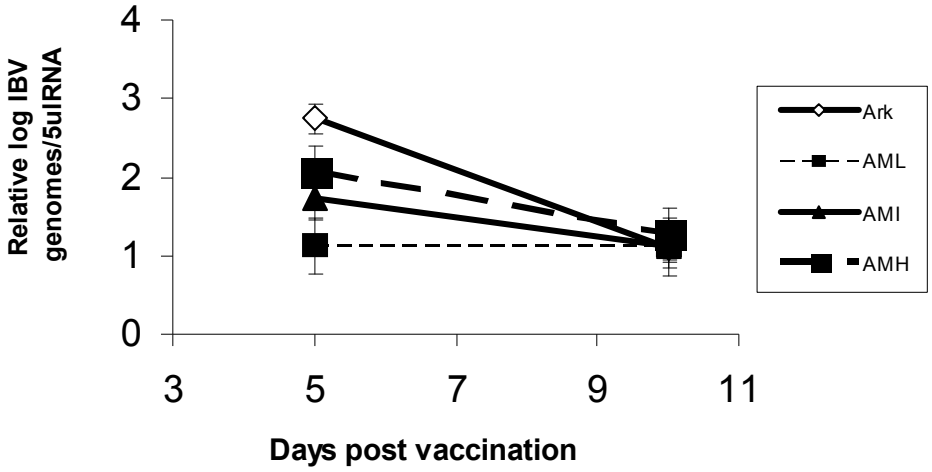

Figure 5. 7: *Ark vaccine genome copy dynamics in the trachea of chickens vaccinated with either Ark alone (Ark) or Ark/Mass vaccines combined (AML, AMI and AMH) between 5 and 10 DPV. Data points indicate mean relative log IBV genomes (n=24).*

Detection of Mass vaccine virus in the lachrymal fluid

Mass vaccine virus was detected in all the chickens that received the Ark and Mass combined in all days evaluated. The Mass genome copy numbers were 10 times higher than Ark vaccine genomes detected in chickens vaccinated with Ark vaccine alone and were not affected by the amount of Ark vaccine combined with (Fig. 5.8). Even in the group that received Ark and Mass combined at equal EID_{50}, Mass vaccine replicated to similar levels as in the two other groups with lower amounts of Ark vaccine, indicating that Ark vaccine did not interfere with replication of Mass vaccine. The genome copy numbers reduced significantly ($P<0.05$) at 10 DPV from those detected at either of the earlier days and were similar to those detected for Ark vaccine in these groups (Fig. 5.8)

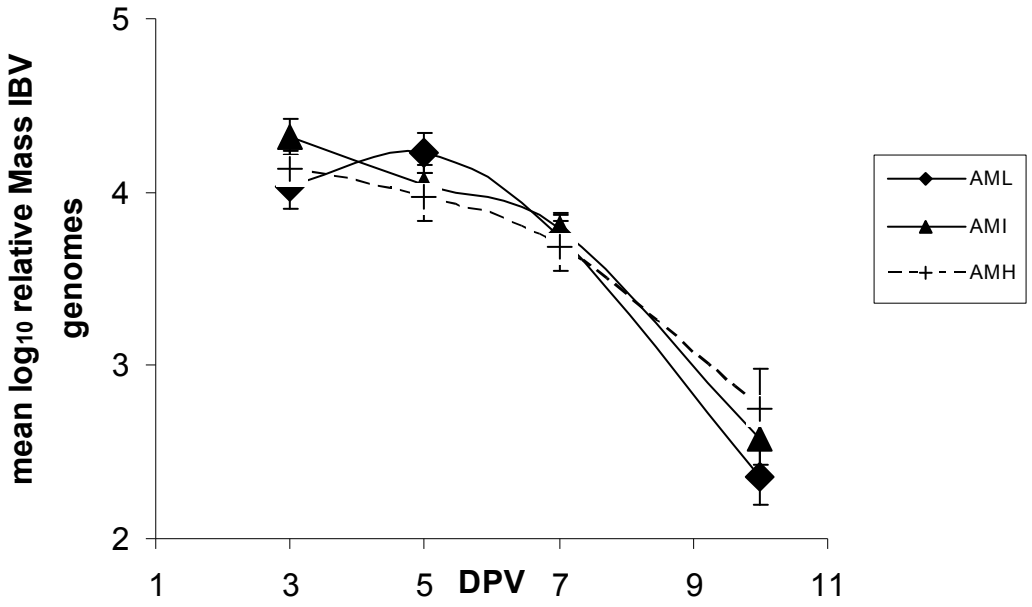

Figure 5. 8: *Relative IBV Mass genome copies 3, 5, 7 and 10 DPV as determined by Mass vaccine specific qRT-PCR in RNA extracted from lachrymal fluid of chickens vaccinated with increasing doses of Ark combined with Mass vaccine. Each data point represents mean log relative genomes (n=18). Error bars indicate SEM.*

Selection of Ark vaccine virus in the presence of Mass vaccine

Our qRT-PCR findings indicated that some Ark vaccine viruses were able to replicate in the trachea of vaccinated chickens in the presence of Mass vaccine albeit at a lower level than Ark vaccine alone viruses. We evaluated the Ark vaccine subpopulations detected in trachea of chickens receiving Ark vaccine alone and those that received a similar Ark vaccine dose combined with Mass vaccines. Previously described Ark vaccine subpopulations C1-C5 and other variants were detected in tracheal samples from chickens vaccinated with Ark vaccine alone and also combined Ark and Mass combined. We found similar subpopulations in both groups but the incidence of detection was

different for some subpopulations between the groups at 5 DPV (Fig. 5.9). Although the difference was not significant, the C5 subpopulation was detected in a higher number of chickens in the Ark alone group than in the Ark and Mass combined groups suggesting that this subpopulation may be less fit to compete with Mass vaccine viruses in the Ark/Mass combined group. In contrast, the C3 subpopulation and its variants were detected at a significantly lower frequency in the Ark alone group as compared to its frequency in the Ark and Mass combined group. This may suggest that the C3 subpopulation may have a better fitness than some other Ark vaccine subpopulation (C5) that were detected less frequently in the vaccine groups combined with Mass vaccine. In both groups the most frequently detected subpopulation was the C4 subpopulation at 10 DPV (Fig. 5.10). In addition, the C4 subpopulation increased in incidence from 5 to 10 DPV in contrast with most other subpopulations that reduced in incidence over the same time period (Fig. 5.10). In particular, the major vaccine population was not detected in any sample at 10 DPV and the C5 found in Ark alone group at 5 DPV reduced sharply by 10 DPV.

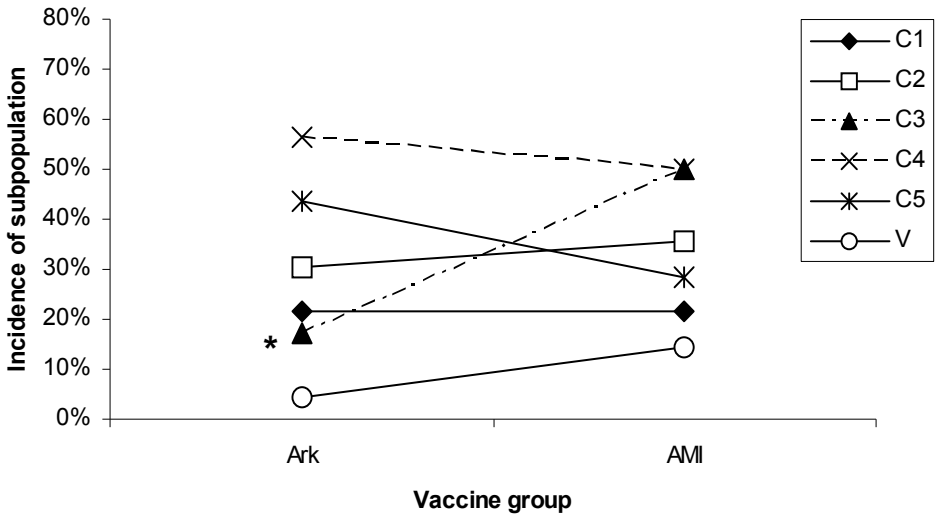

Figure 5. 9: *Incidence of Ark vaccine subpopulations detected at 5 DPV in the trachea of chickens vaccinated with Ark alone (Ark) and those vaccinated with a similar Ark dose combined with Mass vaccine (AMI). Tracheal samples were processed as described in previous chapters for sequencing and chromatogram analysis. *Incidence of this subpopulation in the two groups is significantly different (P<0.05).*

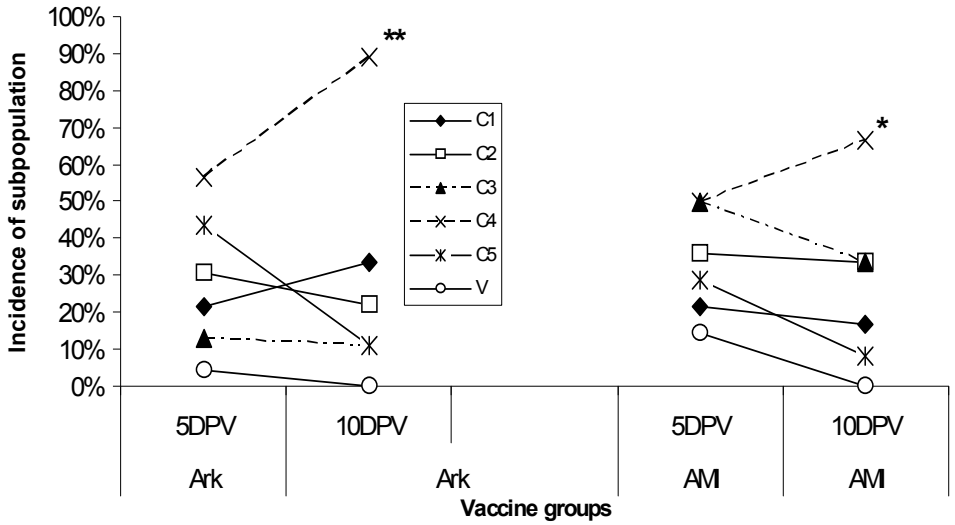

Figure 5. 10: *Dynamics of Ark vaccine subpopulations in the trachea of chickens vaccinated with Ark alone (Ark) and those vaccinated with a similar Ark dose combined with Mass vaccine (AMI) between 5 and 10 DPV. **Incidence of this subpopulation on indicated day is significantly higher than other subpopulations in the same group by Fisher's exact test ($p<0.05$). *Incidence of this subpopulation is significantly different from the subpopulations with the three lowest incidences in the same group on this day.*

Comparing the vaccine subpopulation in the three groups that received Ark and Mass combined (results not shown); we found that C3 was more frequently found in the low dose group than intermediate or high dose group while C4 is more predominant in the high dose group. The major vaccine population was rarely detected even in the low dose group in chickens vaccinated with combined Ark and Mass vaccines.

Discussion

The factors behind high frequency of isolation of IBV similar to Ark vaccine viruses in respiratory cases of poultry despite vaccination remain to be elucidated. We and others previously found that only minor viral subpopulations within Ark vaccines are able to efficiently replicate in the upper respiratory tract of chickens (van Santen & Toro, 2008; McKinley *et al*., 2008). In this study, we found that after combined eye drop vaccination of 2-day-old chickens with Ark and Mass vaccines, no Ark vaccine virus could be detected in quantifiable amounts in the lachrymal fluid of vaccinated chickens through 7 DPV. This is in sharp contrast to the chickens that were given Ark vaccine alone, where we detected Ark vaccine virus in all chickens throughout the sampling period in genome copies over 100 times the assay's reliable quantification lower limit, suggesting interference of replication by Mass vaccine.

We varied the Ark vaccine dose in increasing amounts and combined it with a constant amount of Mass vaccine to determine if increased Ark vaccine dose could overcome this interference. We found that even at the highest dose of Ark vaccine, Ark vaccine virus was still not detectable above the reliable quantification lower limits in lachrymal fluid until 10 DPV. In all groups, Mass vaccine replicated to genome copy numbers above 3000-fold higher than detection limit and no difference was detected in Mass vaccine virus genome copy numbers in the lachrymal fluid of chickens receiving different doses of Ark vaccine combined with Mass vaccine. The interference therefore appears to be one sided. These results indicate that Mass vaccine virus has better replication advantage over the Ark vaccine viruses following eye drop administration. Thus Mass vaccine replication advantage may be interfering negatively with the replication of Ark vaccine viruses early on after administration. We also found that Ark

vaccine started to be detected at low copy numbers in lachrymal fluid at 10 DPV in all the groups that had combined Ark and Mass vaccines. It is interesting to note that this is the time the Mass vaccine virus genome copies declined 10 times lower than those detected at earlier days. This finding further points to the possibility that failure of Ark vaccines to replicate may be due to higher replication of Mass vaccine virus at early stages after inoculation.

Although not investigated in this study, receptor competition has been suggested as a probable cause of vaccine interference in multi strain single valence administration (Vidor, 2007). It is likely that the minor, more fit Ark vaccine subpopulations described earlier may exist in very low copy numbers within Ark vaccine vials, reducing the available infectious viral titer available for efficient replication in the upper respiratory tissues of chickens. Consequently in combined vaccines, the Mass vaccine predominates in replication. Furthermore, in the previous study reported by van Santen & Toro, (2008) no selection of Mass vaccine virus was detected and the virus detected in vaccinated chickens was similar to that found in vaccine vials. This may mean all the viruses contained in Mass vaccine vial are able to efficiently replicate in the chicken, unlike Ark vaccine where only a minor component is able to efficiently replicate.

Our findings corroborate the findings of Alvarado *et al*., (2006) who found that Mass vaccine was detected earlier on and Ark vaccine viruses later after combined administration. They argued that this difference in detection period might be due to difference in attenuation of the vaccines leading to difference in replication.

In the group where we gave the highest Ark vaccine dose, (Ark vaccine in equal EID_{50} with Mass vaccine), we expected to overcome the suppression of Ark replication if

indeed replication of the two vaccines was similar. In the lachrymal tissue, this did not occur and no difference could be seen whether it was low dose or high dose Ark vaccine inoculated as long as it was combined with Mass vaccine, indicating that Mass vaccine clearly replicates better than Ark vaccine in the ocular tissues. Some replication of Ark vaccine was detected in the trachea at 5 DPV. However interference by Mass vaccine was still evident based on the Ark genome copy numbers detected in the low and intermediate groups which were significantly lower than those detected in chickens vaccinated with Ark alone. In our study, interference of Ark replication by Mass vaccine in the trachea was to some extent reduced by increasing the dose of Ark administered. In the group that received Ark and Mass vaccines at equal EID_{50} ($1.5 X10^5$ EID_{50}), the Ark viral load detected at 5 DPV was higher than that detected in the low and intermediate dose groups, and was not significantly different from that detected in the group that received Ark alone. It is important to note that our low dose Ark vaccine group chickens received the equivalent of a single dose of Ark vaccine based on the vaccine vial doses indicated by the manufacturer and which in our study showed very low and in some chickens complete absence of Ark replication in the trachea at 5 DPV.

Inter-serotype interference has also been seen with multivalent Dengue fever vaccines where one serotype viral load (viremia) dominated other serotypes in a tetravalent formulation of Dengue fever vaccine in human subjects (Kanesa Thasan *et al*., 2001) and a monkey model (Guy *et al*., 2009). In both studies the serotype that induced a high viremia also resulted in induction of higher antibodies. In the latter study, reduction of titer administered for the dominant serotype was able to overcome the interference. Winterfield, (1969) first reported interference of induction of neutralizing antibodies

against the IBV Connecticut serotype by the Mass serotype. However no associated reduction in protection against Connecticut was reported.

Failure of vaccine viruses to replicate early may lead to failure to induce timely or optimum neutralizing antibodies. This could result in chickens that are not well protected against field viruses. Another consequence may be persistence of vaccine viruses since clearance of vaccine viruses requires a good primary immune response. In our study we found Ark vaccine viruses starting to replicate and be detected at 10 DPV, meaning that these vaccine viruses were likely to persist in the vaccinated chicken longer than expected. Furthermore, since the Ark vaccine used in this study has multiple subpopulations selected in chickens, failure to clear vaccine viruses may result in selection and persistence of the more fit subpopulations in the vaccinated chickens.

We found that some Ark vaccine subpopulations (C5) were not able to replicate efficiently in the trachea in the presence of Mass vaccine while others (e.g., C3) were found more frequently in the Ark and Mass combined groups than in the Ark alone group. This may point to a difference in the relative fitness abilities of these subpopulations. The C4 subpopulation was detected as the predominant subpopulation in all the vaccine groups. In addition this subpopulation increased in incidence while in general most other Ark vaccine subpopulations decreased in incidence between 5 and 10 DPV. This may indicate that the C4 subpopulation has better fitness than other Ark vaccine subpopulations within the vaccine used in this study. This relatively better fitness for the C4 subpopulation remains to be evaluated, whether it is beneficial for immune induction in chicken or it contributes to pathology in the trachea and/or vaccine virus persistence. Persisting subpopulations in the field may undergo mutations and also offer

genetic material for recombination leading to generation of more virulent variants (van Santen & Toro, 2008; McKinley *et al.,* 2008; Domingo, 1996, 1998).

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CHAPTER 6

CONCLUSION

In the US, despite IBV vaccination with multiple serotype IBV vaccines, field IBV cases continue to be reported. Interestingly ArkDPI type of IBV continue to be the most frequently isolated in these field cases despite extensive use of ArkDPI-derived live attenuated vaccines in combination with other serotype vaccines (Jackwood *et al*., 2005). In addition Ark vaccine viruses have been found to persist in vaccinated flocks and the resulting protection demonstrated to be lower compared to other IBV serotypes (Jackwood *et al*., 2009). The overall objective of this work was to understand the factors behind the high frequency of isolation of ArkDPI IBV despite vaccination. Many factors may affect the outcome of vaccination in poultry flocks, including factors associated with the administered vaccines, methods of administration and/or the chickens themselves (McMullin, 1985). Previous reports indicating a predominance of mainly ArkDPI field isolates despite administration of many other IBV serotypes in addition to ArkDPI-derived vaccines may suggest that inherent characteristics of ArkDPI vaccines themselves may be contributing to the problem. Analysis of ArkDPI vaccines in use in the US today revealed they are heterogeneous and that the degree of heterogeneity differs amongst the different ArkDPI-derived vaccines from different manufacturers (van Santen

and Toro, 2008 & McKinley *et al*., 2008). In addition, minor viral subpopulations within these vaccines were found to replicate better and were selected as early as three days post vaccination (van Santen and Toro, 2008). Analysis of the different ArkDPI-derived vaccines revealed they differ in proportion of these selected minor subpopulations. Our current work has further looked at the significance of the presence of viral subpopulations within Ark vaccines. We hypothesized that the presence within the ArkDPI-derived vaccines of minor viral subpopulations that more efficiently replicate in chickens coupled with the practice of multiple serotype vaccine administration in the field might play a role. Our results suggest several characteristics inherent in ArkDPI-derived vaccines may be playing a role in increased frequency of ArkDPI field isolates and poor protection by ArkDPI vaccines.

In particular, ArkDPI vaccines from different manufacturers result in different vaccination outcomes. These differences correlate with proportion of selectable viral subpopulation for some ArkDPI vaccines. One of the ArkDPI vaccines with high amount of selected viral subpopulations induced a more severe vaccine reaction (respiratory signs and tracheal pathology) while another did not. Another ArkDPI vaccine has a low amount of selectable viral subpopulation but still induced severe vaccine reactions. This latter ArkDPI vaccine also resulted in multiple subpopulations in vaccinated chickens. Temporal evolution of these viral subpopulations through selection occurred in the trachea of vaccinated chickens resulting in persistence of a single vaccine viral subpopulation in most of the vaccinated chickens. Such persisting vaccine viruses may inherently be fit to replicate in the trachea, resulting in clinical IBV disease signs reported in field conditions. This may suggest that some of ArkDPI vaccines may not be fully

attenuated and others may have residual minor viral subpopulations that may still be highly virulent for the chicken respiratory tract. It is also possible that these persisting vaccine viruses may undergo genetic changes over time becoming more virulent too. Furthermore, we also found that these persisting Ark vaccine viruses are more readily passed on to non-vaccinated contact birds than other subpopulations within the vaccine, another factor that may lead to persistence and increased virulence of vaccine viruses in vaccinated flocks through back passage.

We also find that outcome of ArkDPI vaccination may also be affected by the dose of vaccine received. A dose of $4X10^4$ EID_{50} (higher than minimum titre required for IBV vaccine) for two ArkDPI vaccines resulted in relatively poor viral replication, which ultimately resulted in significantly lower systemic antibodies in vaccinated chickens for these two ArkDPI vaccines compared to two others. These two vaccines also have lower proportion of selectable virus, which possibly affected the amount of viral titer able to replicate in the chicken. When we repeated the experiment but used a higher dose (1.5 X 10^5EID_{50}) for one of the vaccines with poor viral replication, relatively high viral load and strong mucosal and systemic antibodies were detected for this vaccine. Since dose variability is a common occurrence during poultry mass vaccine administration, it is possible that use of the low replicating ArkDPI vaccines may ultimately result in suboptimal induction of immune response against Ark serotype viruses in those chickens that receive low titres. However higher doses of some ArkDPI vaccines resulted in selection of some vaccine viruses that unfortunately were also found to persist in the respiratory tract. A balance of the right dose increase would therefore have to be

determined which would result in enhanced immunity but no selection of persisting vaccine viruses.

Replication of ArkDPI vaccine was also negatively affected by presence of competing Mass serotype vaccine strain. Suppression of replication was more prominent when Ark vaccine was co-administered with Mass as a single dose based on vaccine vial number of doses. Suppression of Ark vaccine virus replication appeared to be more severe early on after vaccination since some Ark vaccine virus could be detected at 10 DPV. An increased dose of Ark vaccine overcame substantial suppression by Mass vaccine. This further suggests that the amount of Ark vaccine virus able to replicate in the chicken may be contributing to the poor protection against Ark challenge viruses especially when other IBV serotype vaccine viruses with low or no cross-protection are simultaneously included during vaccination. Poor primary replication of Ark vaccine viruses may also lead to inability to clear Ark vaccine viruses further contributing to Ark vaccine virus persistence.

Based on our findings, further analysis of some viral subpopulations within some ArkDPI vaccines for residual virulence (potential to cause pathology in the respiratory tract) needs to be carried out in their cloned state. In addition, for three of the current ArkDPI vaccines, increasing the dose of the vaccine may be able to improve the outcome of vaccination.

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