DEVELOPMENT OF DIAGNOSTIC METHODS AND EXAMINATION OF VACCINATION FAILURES FOR AVIAN CORONAVIRUS INFECTIOUS BRONCHITIS VIRUS

by

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(Under the Direction of MARK W. JACKWOOD)

ABSTRACT

Infectious bronchitis virus (IBV) is an avian coronavirus with major economic importance to commercial chicken producers worldwide. Due to the existence of multiple serotypes and variants of the virus that do not cross protect, it is essential to choose the right vaccine types and to establish an optimized vaccine protocol using rapid diagnosis of circulating viruses. Thus, rapid, sensitive and specific diagnostic tests that can distinguish different IBV types are extremely important. In addition, understanding the dynamics of IBV vaccines in poultry flocks where mass vaccine delivery methods are used is also a key to control.

In an effort to improve currently used diagnostic tests, a microsphere-based assay was developed and evaluated for simultaneous detection of the five most common IBV serotypes in the USA; Arkansas (Ark), Connecticut (Conn), Massachusetts (Mass), Delaware (DE072), and Georgia 98 (GA98). The microsphere-based assay was highly specific and able to detecte co-infections in clinical samples, which was an advantage over currently used tests. These results demonstrate that the microsphere-based assay is a rapid and accurate diagnostic tool with the potential for high throughput.

To understand the dynamics and persistence of Arkansas vaccine, we evaluated vaccine interference in one-day old broilers vaccinated in a spray-cabinet and we examined efficacy of different vaccine application methods. There was no interference between vaccines; rather there was a slight enhancement of protection against the Ark challenge virus when combined with other IBV vaccine types. Our findings suggested that Ark vaccine virus was not providing adequate protection against homologous challenge when it was administrated via spray cabinet or drinking water. Moreover, detection of IBV vaccine virus early after administration (regardless of strain or route) correlated with protection against homologous challenge, which may be a good indicator of vaccine efficacy in the field since humoral antibody titers are typically low or undetectable following vaccination. These experiments provide some key findings that can be used to direct the efforts for improving the efficacy of IBV Ark type vaccines given in the hatchery and are an important step in elucidating the factors contributing to the persistence of Ark vaccine in the field.

INDEX WORDS: Coronavirus, avian infectious bronchitis virus, microsphere-based assay, serotype, Ark-DPI, Massachusetts, Connecticut, Delaware 072, Georgia 98, qRT-PCR, vaccine, vaccine interference, spike gene sequence, subpopulation

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DEDICATION

To my parents, Hyunkil Roh and Younghee Oh, for their endless love, continuous support and encouragement throughout my life

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CHAPTER 1

INTRODUCTION

Avian infectious bronchitis virus (IBV) is a gamma coronavirus, which belongs to the family Coronaviridae. IBV is an enveloped positive sense RNA virus causing infectious bronchitis (IB), a highly contagious upper-respiratory tract disease in chickens that results in huge economic losses to the U.S poultry industry by affecting the performance of broilers, layers and breeders. Live attenuated vaccines are the main approach for controlling IBV, but rapid mutation of the viral RNA genome results in numerous serotypes, which are poorly cross protective. For effective control, it is essential to choose the right vaccine types using rapid diagnosis of circulating viruses and to establish an optimized vaccine protocol. Thus, rapid, sensitive and specific diagnostic tests that can distinguish different IBV types are extremely important. In addition, understanding the dynamics of IBV vaccines in poultry flocks where mass vaccine delivery methods are used is also a key to control.

Objective 1 - Develop a rapid, accurate and high throughput diagnostic assay for detection of major U.S. IBV strains. Our working hypothesis was that a rapid identification of circulating field strains using an effective diagnostic tool would help to improve vaccination strategies in the field. A microsphere-based assay, which is high-throughput and less-time consuming when compared to other IBV diagnostic tests, was used. The capability of multiplexing this assay for up to 100 analytes also allows detection of multiple viruses simultaneously. We designed nucleotide probes for targeting serotype specific regions of the

IBV S1 gene for the most common IBV serotypes isolated in the U.S. Our target serotypes were: Arkansas, Connecticut, Massachusetts, Delaware and GA98. We evaluated this assay and examined its suitability for processing high numbers of field samples in a relatively short time.

Objective 2 - Develop a rapid, and type specific quantitative technique to detect and differentiate IBV strains for use as a research tool to study IBV vaccination. Quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) is a rapid, accurate and quantitative method for detecting small amounts of RNA. Previously, a universal taqman probe targeting all infectious bronchitis viruses was designed in our laboratory. We further designed a minor grove binding (MGB) taqman probe for each of the serotypes: Arkansas, Connecticut, Massachusetts, and Delaware/GA98. By multiplexing these probes, we were able to simultaneously detect and quantify multiple viruses existing in a sample.

Objective 3 - Examine the dynamics of IBV vaccine infection and replication in commercial poultry to better understand coverage patterns and vaccine efficacy. Our working hypothesis was that negative vaccine interactions are affecting the efficacy of the Arkansas vaccine in commercial broilers. We vaccinated one-day old broilers (with maternal antibodies to IBV) with commercially available multivalent IBV vaccines (which include the Arkansas vaccine), and determined the level of virus in the trachea and clearance or persistence of the vaccine viruses to assess the level of interference. We also tested different vaccine application methods, different volumes and various doses to determine factors affecting the efficacy and persistence of the Arkansas vaccine. This study provided valuable data that can be used to improve the current vaccination methods, which will help to prevent and control infectious bronchitis.

CHAPTER 2

LITERATURE REVIEW

PART I

Overview of Coronaviruses

1. Classification

Coronavirus is a member of the family *Coronaviridae* within the order *Nidovirales*. Coronaviruses possess a linear, positive sense, single-stranded RNA genome that is the largest among known viral RNA genomes, ranging from 26 to 32kb. Coronaviruses have round, enveloped virions approximately 100 to 160nm in diameter with surface spike structures that give them a distinct crown-like shape.

Coronaviruses are presently divided into three groups (alpha, beta, and gamma) and each group is further divided into subgroups. The group classification was based initially on the cross reactivity of neutralizing serum. Since whole genome sequences are now available, phylogenetic analysis and genome structures are used for grouping. Alphacoronaviruses (group 1) contain feline infectious peritonitis virus (FIPV), canine coronavirus (CCoV), porcine transmissible gastroenteritis virus (TGEV), human CoV-NL63 (HCoV-NL63) and human CoV-229E (HCoV-229E). Betacoronaviruses (group 2) contain human CoV OC43 (HCoV-OC43), mouse hepatitis virus (MHV), and bovine coronavirus (BCoV). Recently, SARS-CoV has been classified as a group 2 coronavirus (2b) after extensive phylogenetic and viral enzyme studies (196).

Gammacoronaviruses (group 3) are mainly avian coronaviruses, including infectious bronchitis virus (IBV), and turkey coronavirus (TCoV), although recently mammalian gammacoronaviruses have been identified. The first reported mammalian gamma CoV (SW1) was discovered in 2008 in liver tissue of a dead beluga whale and its phlyogenetical relationtioship with IBV was revealed (158). Also, Asian leopard cat CoV (ALC-CoV) was discovered and identified as a gammacoronavirus member by phylogenetical analysis of envelope, membrane, and nucleoprotein structural proteins and the two conserved replicase domains (RNA-dependent RNA polymerase and RNA helicase) (70).

2. Viral genome structure and proteins

Viral genome structure

Coronaviruses contain a single positive stranded RNA genome that is 27 to 32 kb long, the largest known genome among RNA viruses. Coronavirus genomic RNA resembles a eukaryotic messenger RNA, contains a 5' cap and a 3' poly-A tail structure, and is infectious itself (149, 186). The 5'end of the coronavirus genome contains a leader sequence of 65 to 98 nucleotides in length. This leader sequence is also found at the 5' end of all viral subgenomic mRNAs. Untranslated regions (UTRs) of 200 to 400 nucleotides exist right after the 5' leader sequence and at the 3' end of viral genome. The 3' end UTR is followed by a poly-A tail. The UTRs at 5' and 3' termini play important roles in genome replication and subgenomic mRNA synthesis (157). All coronaviruses contain five essential open reading frames (ORFs). The viral genome encodes the viral replicase complex [Pol] (ORF1a and b) and structural proteins, which consist of the spike [S], envelope [E], membrane [M] and nucleocapsid [N] proteins. The order of these ORFs in the viral genome is 5'-Pol-S-E-M-N-3'.

Nonstructural proteins (NSPs)

The ORF1 a/b comprises almost two–thirds of the genome. ORF 1a and 1b overlap in a small area between the ORF 1a 3'terminal and ORF1b 5' terminal and translation of these 2 ORFs is directed by a -1 ribosomal frame shift (RFS). Translation of ORF1a and ORF1ab (by RFS) gives rise to 2 large polyproteins which undergo posttranslational cleavage by cis-acting viral enzymes (97). Autoprocessing of polyprotein 1a (pp1a) and polyprotein 1ab (pp1ab) releases 16 mature nonstructural proteins including the viral RNA-dependent RNA polymerase (RdRp) (nsp1-nsp16) in all coronaviruses except IBV which lacks nsp1 therefore encoding nsp2-nsp16. Exact functions of nsp1 and nsp2 are not clear, but they are thought to be potential regulatory proteins involved in viral-host interactions. Also, a role in viral replication has been suggested, which is based on experiments with nonviable MHV mutants containing an nsp1 deletion (17). Two Papain-like proteases ($PL1^{pro}$ and $PL2^{pro}$), which are part of nsp3, cleave two or three sites at amino-terminal pp1a/pp1ab regions (nsp1, 2, and 3). In IBV, $PL1^{pro}$ is proteolytically defective and only $PL2^{pro}$ is active, and thus called PL^{pro} (226). In MHV, experimental deletion of $PL1^{pro}$ generates mutants with severe growth deficiencies (100). In addition to its protease activity, PL^{pro} of SARS-CoV and $PL2^{pro}$ of HCoV-NL63 negatively regulates antiviral defenses of the cell by disrupting IFN-induction signaling (198). A picornavirus 3C-like proteinase/ main protease domain ($3CL^{pro}/M^{pro}$) residing in nsp5, flanked by transmembrane-domains (nsp4, nsp6), cleaves most of the sites in pp1a/pp1ab except sites between nsp1, 2, 3 and 4 (225). The Nsp4 transmembrane (TM) domain may be involved in anchoring the viral replicase to intracellular membranes. In an experiment using a temperature sensitive MHV, nsp4 was shown to be essential for the assembly of a functional replicase-transcriptase complex and is required for RNA synthesis (184). It has also been shown that nsp4

plays an important role in generating a membrane-associated replicase complex by serving as an anchor (35). Nsp6 of IBV, a multiple-spanning transmembrane protein located to the endoplasmic reticulum (ER), activates autophagy of the infected cell (48), however the exact function of nsp6 in virus replication has not been identified. In a recent SARS CoV study, it has been shown that nsp8 is an additional RdRp that may produce the RNA primers required for the primer-dependent RdRp activity associated with nsp12 (RdRp) (114). In FCoV and HCoV229E, an nsp7 and nsp8 complex (2:1 heterodimer) also had RNA polymerase activity (219). Nsp7, 8, 9, and 10 are interacting with other proteins from the pp1a/pp1ab region and this protein-protein interaction can be crucial for RNA synthesis. Nsp9, which forms a dimer, is shown to be essential in viral replication of SARS-CoV (159). Nsp11 is located upstream of the frame shift site but its exact role has not been studied. Nsp12 contains the RNA dependent RNA polymerase domain at its carboxyl-terminal end and interacts with other replicase proteins (16). Nsp13 contains a helicase domain and a zinc-binding domain (ZBD). The ZBD is involved in viral replication by modulating helicase enzymatic activity (190). Furthermore, nsp13 has an enzyme activity that might be involved in the 5'-capping reaction of coronavirus. Nsp14 contains a 3'-to-5' exoribonuclease (ExoN) that might be involved in proofreading activity during coronavirus RNA replication. In addition, the ExoN domain has been shown to be involved in coronavirus replication and subgenomic mRNA synthesis (160). It has been shown that nsp15-associated NendoU is required for virus replication, but the exact function is still not clear. The nsp16 of SARS-CoV was shown to contained a methyl transferase (MT) domain that is essential for viral replication and its putative function in production of the cap structure has been suggested (213).

S protein

The S protein, translated from subgenomic(sg) messenger RNA2, forms spikes projecting from the viral surface, which gives a crown-like shape to the virus. It is a highly glycosylated protein with a molecular mass of 160 to 180 kd. In beta- and gammacoronaviruses, spike proteins are cleaved into an amino terminal S1 subunit and a carboxyl terminal S2 subunit by host cell serine proteases. The head of the S protein is formed by the S1 subunit and is responsible for attachment of the virus to cell receptors. The S2 subunit is anchored in the virus membrane and associated with virus-host membrane fusion.

The S protein determines the host/tissue tropism because it binds to specific receptors on the cell surface, and the amino acid sequence of the S1 receptor-binding domain is critical for binding of virus to the target cell receptor. Using a reverse genetic system, Casais *et al.* replaced the Beaudette spike gene with that of the M41-CK strain and confirmed that cell tropism of IBV is determined by the S protein (22). In another study, TGEV mutants lost their enteric tropism when only two amino acid changes were made in the receptor binding domain of the S1 region (9) demonstrating a critical role for the receptor-binding domain of the S protein in virus binding. Although cellular receptors for most coronaviruses, including IBV, have not been identified, receptors for some coronaviruses including MHV, TGEV, and SARS-CoV have been elucidated. The cellular receptor of MHV is a carcinoembryonic antigen-cell adhesion molecule (CECAM), a type I transmembrane protein belonging to the immunoglobulin superfamily. HCoV-229E, TGEV, CCoV and FIPV all use the aminopeptidase N (APN) protein, which is expressed on epithelial cells of the respiratory and enteric tract (reviewed in (10)). SARS-CoV (145) and HCoV-NL63 (194) use angiotensin-converting enzyme 2 (ACE2) as a receptor, which is abundant in lung tissues. Some betacoronaviruses (BCoV and HCoV-OC43) bind to sialic acid

on the cell surface (10). As for IBV, sialic acid (217) and heparin sulfate (151) have been suggested but the exact host cell receptor is yet to be defined.

The S protein also contains multiple epitopes that induce neutralizing antibodies. The S1 region, especially the hypervariable domain, has neutralizing antibody inducing epitopes in IBV (29). It was reported that IBV does not induce neutralizing antibodies without the S1 region (28), however the SARS-CoV S2 region contains an epitope recognized by human B1 cells (71) and is able to induce neutralizing antibodies against a pseudo-SARS-CoV spike (224). In SARS-CoV, multiple conformation-dependent epitopes in the S1 region have been identified and induce neutralizing antibodies (103).

The S protein can be an important factor affecting the pathogenicity of coronavirus since binding of S protein to cell surface receptors is the first step in virus infection. But S protein alone is not sufficient for pathogenicity to be fully expressed in IBV. Exchange of the S protein between an attenuated IBV strain and a pathogenic strain did not restore the pathogenicity of the attenuated IBV strain (107), and it has been suggested that other replicase genes are more important in determining pathogenicity (8).

E protein

The E protein is a small integral membrane protein with an approximate size of 9-12 kd. It only comprises a minor portion of virus structure but plays an important role in virus assembly. In IBV, the E protein is produced from sgmRNA3. The E protein is localized to the ER or Golgi complex during virus replication and translation (47). The exact roles of E protein have yet to be identified, but it is recognized that the E protein is required for virus particle formation and budding (47) and interacts with M protein to form virus particles (208). An MHV mutant with an E protein defect showed alteration of viral morphology in an experimental setting confirming

that E protein contributes to the integrity of the viral structure (84). Also the role of the E protein as an inducer of apoptosis has been identified in MHV-infected cells (7).

M protein

M proteins are integral membrane proteins translated from subgenomic mRNA4 and, through interaction with N, E and S proteins, play a role in initiating virus particle assembly (157). Also M protein has been found in the viral internal core along with N protein in TGEV (180), and its potential role in viral nucleocapsid formation and core stability via interaction with N protein has been suggested (78). The M protein of some coronaviruses such as TGEV can induce interferon-alpha (142).

N protein

The N protein is a multifunctional phosphoprotein that is translated from subgenomic mRNA6. The N protein contains 3 conserved domains; two independent RNA binding domains (an N terminal domain (NTD) and a C terminal dimerization domain (CTD)) and a cytoplasmic domain for interaction with M proteins. As a structural protein, it interacts with viral genomic RNA and forms the viral helical ribonucleoprotein. It has been shown that the CTD binds to an RNA packaging signal (110) and plays a principal role in the helical packaging process (33). In addition, N protein plays an important role in viral RNA synthesis and translation, identified in TGEV and SARS-CoV, which is important for correct folding of the newly synthesized viral genome (229). It is also proposed that the chaperone activity of the N protein is important in template switching during viral transcription (228). Recently it has been suggested that the NTD interacts with the transcriptional regulatory sequence (TRS) and has a helix melting activity which plays an important role in sgRNA synthesis in MHV (101). The N protein interacts not only with negative and positive sense viral genomes, but also with all sgRNAs including the 5’

leader (171). In addition, N protein contains a M protein binding domain which forms protein-protein interactions with M proteins, which leads to the incorporation of the nucleocapsid into the progeny virion (169). The N protein is also associated with cell cycle arrest (218) and apoptosis (199) in the infected cell.

Accessory proteins

In addition to ORF 1 and the structural protein genes, several ORFs encoding additional non-structural genes exist. Numbers, nucleotide sequence and gene order of these ORFs vary among coronaviruses, but are generally conserved within the same group hence the name group-specific proteins. The exact functions of most of these proteins are yet to be discovered, however some of the proteins are dispensable for virus replication in cell culture and are therefore called accessory proteins (181). Briefly, TGEV, an alphacoronavirus, contains two accessory ORFs (3a and 3b) between the S and E protein genes, as well as an ORF (gene 7) at the 3' end of the N protein gene. Betacoronaviruses (e.g. MHV) have two accessory ORFs (2a and 2b) located between ORF1b and the S protein gene, and two more accessory ORFs (4 and 5a) between the S and E protein genes. The ORF2b encodes the hemagglutinin-esterase (HE) protein, an accessory structural protein specific to betacoronaviruses. The HE protein is non-essential for virus replication in cell culture, but believed to play a role in viral infectivity and virulence by mediating attachment to sialic acid residues on the cell surface (134, 147). The genomic organization of gammacoronaviruses, which includes IBV and TCoV, follows; 5'UTR-ORF1a/1ab-S-3a/b-E-M-5a/b-N-3'UTR- poly A tail. In an *in-vitro* setting, neither the 3a or 3b gene products of IBV were essential for replication of virus or the initiation of internal translation of E protein (106, 191). Similarly the gene products of ORF5 a/b were dispensable for replication of recombinant viruses of the IBV Beaudette strain *in-ovo, in-vivo* and *ex-vivo*(21).

3. Viral Replication and Transcription

The first step in virus replication is the binding of the virion to cellular receptors using the S protein. Virus-cell membrane fusion occurs in either a pH dependent manner (fusion occurs in endosomal vesicles) or a pH-independent manner (at the cell surface) (170). After virus-membrane fusion, the viral genomic RNA (gRNA) is released into the cytoplasm. Upon release into the cytoplasm, the positive sense viral gRNA serves as mRNA due to its 5' cap structure and initiates translation of the ORF1a and ORF1ab proteins (replicase-transcriptase polyproteins) to initiate virus replication. Binding of cellular initiation factors to the 5' cap structure of gRNA followed by recruitment of the ribosomal complex initiate viral translation (181). ORF1a and ORF1b briefly overlap and translation of ORF1 is regulated by ribosomal frame shifting. More than one-third of the ribosomes shift their reading frame when they encounter the frame shift signal near the ORF1a/1b junction and this mechanism ensures that the ORF1b derived replicase proteins are produced in smaller amounts (67, 181). Potential frame shifting signals are the heptameric slippery sequence and the RNA pseudoknot structure in gRNA. The slippery sequence is located at the actual -1 frameshift and has a UUUAAAC sequence (XXXYYYN motif), which is conserved in all coronaviruses. The slippery sequence itself, however, is not sufficient for ribosomal shifting. The RNA pseudoknot is an RNA structure which consists of at least two stem-loops with another stem-loop intercalated between the first two (176). It is believed that the RNA pseudoknot, located 5-7 nt downstream of the slippery site, results in a ribosomal pause, which allows its realignment of tRNA on the mRNA in a new frame. This results in the slippage of tRNAs within ribosomal aminoacyl and peptidyl sites into -1 frame

(150). Recently, it has been found that a stem-loop structure can substitute for the pseudoknot structure in -1 RNA frame shifting (222).

Coronavirus gRNA also acts a template to generate negative stranded genomic and subgenomic RNAs. The switch between gRNA serving as template for translation and gRNA serving as template for replication is controlled by the communication of two switching signals located in 5’ and 3’ UTRs of gRNA (77). The viral RdRp complex composed of PP1a and PP1ab is involved in this process.

Among suggested models of coronavirus transcription, the discontinuous extension of minus-strand RNA synthesis seems most applicable for coronavirus subgenomic mRNA (sgmRNA) synthesis (183). In this suggested model, instead of sgmRNA derived directly from genome-length negative stranded RNA, subgenome-length negative strand RNA is first generated in a discontinuous manner using the genome as a template and subsequently positive sense sgmRNAs are transcribed directly from these subgenome-length negative strand RNAs. The positive sense sgmRNA cannot replicate to generate negative sense sgmRNA. Therefore sgmRNAs can only be synthesized from the subgenome-length negative strand (32).

Replication of the coronavirus genome requires a full uninterrupted negative strand RNA. However, during full-length negative strand synthesis, leader switching was possible in an experimental setting using defective interfering (DI) RNA. Therefore, genomic RNA replication also occurs in a similar manner as a discontinuous transcription. Additionally, for genomic RNA replication, 5’ UTR and 3’ UTR sequences for secondary RNA structures are required (181).

Part II.

Infectious Bronchitis Virus

1. Introduction

Infectious bronchitis (IB) or avian infectious bronchitis is a highly contagious viral respiratory disease of chickens caused by infectious bronchitis virus (IBV). The primary targets of the virus are epithelial cells in the respiratory tract, thus IB is characterized by respiratory signs including tracheal rales, coughing, sneezing, and gasping. The virus also replicates in epithelial cells of the oviduct, causing decreases in egg production and egg quality, and in epithelial cells in the kidney resulting in nephritis and mortality. The disease has a major economic impact on the poultry industry through poor weight gain and feed efficiency, condemnation in meat processing and drops in egg production. Although mortality caused by IBV alone is not significant, high morbidity and secondary infections with bacteria or other viruses lead to complication of the disease and increase the mortality rate in chickens, especially in broilers (30).

The virus was first described by Schalk and Hawn in the USA in 1931 as a disease in chicks with clinical signs of gasping and listlessness, reviewed in (81). The infectious agent was not identified and easily confused with a form of infectious laryngotracheitis (ILT), which is an upper respiratory disease in chickens caused by a DNA virus. In 1936, Beach and Schalm cleared up this confusion by providing evidence through cross-immunity studies showing that IB and ILT are caused by distinct agents. In 1950s, commercial IB vaccine became available. The Massachusetts serotype was the only type identified until the first demonstration of the Connecticut isolate, which produced similar disease but showed antigenic differences from the

Massachusetts isolate, described in a report by Jungherr and his colleagues in 1956 (30). Currently, IBV exists ubiquitously and many different serotypes have been identified within the United States and other countries. The emergence of new variants and serotypes of IBV along with outbreaks of IB, which are often found to be a distinct serotype from the vaccine type, are not uncommon.

2. Pathogenesis of IBV

Depending on dose and inoculation route, IBV has an incubation period of 18 to 36 hours (30). It rapidly spreads in the flock via aerosol droplets, nasal excretions and feces. The initial replication site of IBV is epithelial cells and mucus-secreting goblet cells of the upper respiratory tract. The first description of infectious bronchitis (185) was in chicks in the U.S. Respiratory signs associated with IBV infection include coughing, rales, sneezing, nasal discharge and gasping (30). There may be swollen sinuses, watery eyes, depression and poor weight gain related to IBV infection (30). Morbidity is high, but mortality is generally low unless there is secondary bacterial or immune suppressing viruses involved. Damage to the respiratory tract after infection, results in birds becoming vulnerable to secondary bacterial infections, which can be the main cause of IBV related mortality (25, 207). The severity of disease is much higher in young chicks, less than 2 weeks old, and decreases as they age. Replication of IBV in the trachea reaches peak titer between 3 and 10 days post infection (4, 108)

Within 18 hours of infection, epithelial cells in the trachea become edematous, ciliated cells are lost and the lamina propria becomes mildly infiltrated with heterophils and lymphocytes (30). Regeneration of epithelium is accompanied by a massive infiltration of lymphoid cells in

the lamina propria resulting in the formation of germinal centers. This process starts within 48 hours of infection and the germinal center may be present even after seven days (30).

In addition to respiratory tissues, IBV also replicates in epithelial cells in the kidney, oviduct, testes, harderian gland, and alimentary tract, including the ileum, duodenum, jejunum, cecal tonsils, and cloaca (178). Replication of IBV in enteric tissues normally does not present any clinical signs. Most IBV strains grow in kidney cells of experimentally inoculated embryonated eggs, causing lesions accompanied with the formation of urates. However some strains of IBV are nephropathogenic causing nephritis in chickens, which can be accompanied by high mortality. Chen *et al* (1996) showed the primary replication sites of IBV in the kidney were epithelial cells of the collecting ducts, collecting tubules, distal convoluted tubules and Henle's loop. Kidney failure can occur within 6 to 16 days post infection and presentation of initial respiratory signs (178). Severity of macro and microscipic lesions in the kidney and detection of high IBV titers in the kidney do not always correlate (4). The Australian T strain, isolated from Australia in 1962, was first the documented nephropathogenic IBV strain (reviewed in (51)), and later more nephropathogenic strains were reported in the U.S, Europe, Asia and Africa (14, 20, 34, 99, 156, 227)

Epithelial cells of the reproductive tract in young chicks and laying hens can also be infected by IBV, which results in poor quality eggs, particularly relating to shell quality, and a decrease in egg production (30, 131, 178, 189). Infected oviduct epithelium and glandular cells become edematous, loss of ciliated cells and infiltration of the lamina propria with inflammatory cells generally occurs (30). The severity of these changes in the oviduct and the decrease in egg quality and production may vary based on the IBV strains and the age of the hen (30, 178). Infection of the oviduct in young chicks can cause permanent damage to the oviduct resulting in

so called false layers. In males, epididymal lithiasis and a decrease in fertility was reported in roosters both vaccinated (12, 115) and naturally infected with IBV (12, 210). Also, in experimentally infected roosters, IBV was detected in testicular tissues without causing any pathologic lesions (6, 86).

In addition, IBV can infect lymphoid organs including the Harderian gland (178, 203), a small lymphoid organ located in the eye-socket, and the bursa of Fabricius (4).

It has been shown that virus can still be isolated from the cecal tonsils at 14 weeks after infection and from feces at 20 weeks after infection (1, 30). Also, re-excretion of virus from trachea and cloaca occurred in an experimental setting at the onset of egg production, which is about 19 weeks of age (5, 133).

3. Immune responses to IBV

Among the four structural proteins of IBV, S protein is the major antigen inducing neutralizing antibodies as well as the cell mediated immune response in chickens. It has been shown that the S2 portion of S (162) and the N protein may have epitopes (113, 187) for cross-reactive antibodies but the exact role of these proteins in protection against IBV is not clearly understood.

Most research on IBV immunity has focused on the humoral immune response by measuring antibody levels in serum, lachrymal fluid (87), and trachea washes using the ELISA, virus neutralization (VN) or hemagglutination inhibition (HI) tests (30, 59). Upon IBV infection, there is a primary IgM response which peaks then declines before the IgG response (153). IgG can be detected within four days of initial infection. The initial detection of IgG is normally followed by a peak titer at 21 days post infection and subsequent slow decrease in titer, which

may still be detected for several weeks (153, 178). The IgM response to a second infection (or a boost vaccination) peaks at the same time as IgG but declines rapidly (30). IgG is commonly detected by ELISA or HI tests and used to determine the protection level of birds; however, IgM can be useful for diagnosis of recent IBV infection since it can be detected earlier (153). These immunoglobulin responses are considered to be critical for protection against IBV infections as experimentally bursectomized chickens or B-cell depleted chickens that were vaccinated showed higher clinical signs and kidney lesions (178). However serum antibody levels and protection against IBV does not always correlate (112, 177)

In addition to a systemic immune response, local immunity in the upper-respiratory tract was demonstrated to protect against IBV infection (96). Immunoglobulins associated with local immunity against IBV infections are IBV-specific IgA and IgG, detected in lachrymal fluid (44, 64, 202) in washes from the trachea, oviduct, gut and cecal tonsils (179). IBV-specific IgA secreting cells have been found in the Harderian gland and cecal tissues of chickens using the enzyme-linked immune-spot (ELISPOT) assay (205). The role of the Haderian gland in protection against IBV infection was experimentally shown in chicks where Haderian glands were removed resulting in a decrease in protective immunity following challenge 3 weeks post vaccination (56). While some studies showed that there is no significant correlations between serum or tear antibody levels and the protection level against IBV (90, 163, 220), other studies have shown the association of lachrymal IBV-specific IgA and protection against IBV, indicating IBV-specific IgA as a good measure of protection (44, 202).

Cellular immunity against IBV has been determined to be a critical immune response for initial viral clearance and decreased clinical signs (13, 125, 174, 188). Following initial IBV infection, a significant increase in the Cytotoxic T cell (CTL) response can be found, which

peaks by 10 days post infection. A decline in $CD8^+/CD4^-$ cells correlates with the decline of viral load indicating its role in clearance of the virus (30). In an experimental setting, adoptive transfer of IBV specific $CD8^+$ memory T cells provided protection against disease in chicks following IBV challenge (174).

4. Genetic and antigenic diversity of IBV

The first reported variant of IBV was the Connecticut (Conn) isolate in the U.S which was reported by Jungherr and his colleages in 1956 (reviewed in (62)). This isolate showed no cross-protection or cross-neutralization against the original IBV Massachusetts (Mass) isolate. However according to the study by Jia and his colleagues (128) using monoclonal antibodies and molecular analysis of S1 region of the S gene, there were non-Mass IBV isolates already existing in the U.S as early as 1940.

IBV exists as many serotypes which differ from 2 to 3% up to 50% of S1 amino acid residues, with most serotypes differing by 20-25% of S1 amino acids (23). Point mutations, insertions, deletions and recombinations in the IBV genome, especially in S gene, result in many serotypes of IBV (82, 93, 118, 144, 155, 206). Some IBV strains, such as Mass, exist worldwide while others are limited to a particular geographical area. The reasons why some of the variants distribute widely are still unknown.

In the U.S, many new IBV isolates have been identified since the 1970s based on serological tests (62). Among many identified serotypes, Mass, Arkansas (Ark) (83), and Conn are the most widely distributed and vaccinated against in the U.S. Combined vaccines of these serotypes have proven to provide adequate protection against other IBV variants as well as homologous IBV challenge viruses (2, 122). Despite this, Ark type IBV continues to be

problematic, even under the extensive vaccination protocols used in the south and east, including Georgia and Delmarva regions. Another important IBV isolate is Delaware (DE) 072, which was first reported in 1992 by Gelb et al.(89). The S1 gene of DE072 was shown to share more similarity with the Dutch variant, D1466 (143) than with other US variants. Lee and Jackwood (144) identified a new IBV variant, GA98, which is closely related to DE072 and provided adequate protection against DE072 as well as a homologous challenge after two vaccine applications (118). New variants such as GA08 (121) and California isolates (122) continue to emerge.

Until Dawson & Gough reported IBV variants in the UK in 1971, the Mass serotype was considered the only important IBV in Europe. In 1984, four IBV serotypes were isolated from IB outbreak cases in Mass-vaccinated commercial flocks in the Netherlands (55). These IBVs were new serotypes that were able to break through immunity induced by existing IBV vaccines, and they were designated D207 (or D274), D212 (or D1466), D3896 and D3128. Using tracheal organ cultures (39, 41), more new serotypes of IBV were identified in European countries (62). The most predominant IBV types in Western Europe are the Mass type and 793B type, which emerged in the UK and was reported by Parsons *et al.* in 1992 (173). The 793B type is found everywhere except the US and Australia and live attenuated vaccines (4/91 or CR88) are used in combination with a Mass type vaccine (H120) to provide a wide range of protection against different IBVs (43). Another important IBV serotype in Europe is the QX-like virus (D388), which was first detected around 2008 (99) and has almost 100% similarity to the S1 gene of the original QX strain isolated in China (223). The QX type virus causes a severe disease including nephritis in young birds, high mortality, respiratory distress in broilers and drops in egg production in breeders and layers (false layer syndrome) (62, 132) and remains a major

economic concern in IB-vaccinated flocks in Europe. Currently no commercial vaccine for the QX type virus is available. In China, Mass type IBV was mainly found and Mass type vaccine had been successfully used by the mid 1990s. However currently many additional variants have been found in China such as QX, Q1, Korean variants, Australian T strain, as well as indigenous China variants (62). In South America, the Mass type IBV was first reported in Brazil by Hipolito and his colleagues in 1957 and Ark type was reported in 1986 by Branden and Da Silva (62). Using S1 gene analysis, many different genotypes have been identified in Brazil (164, 211, 212) and unique indigenous IBV strains and the 4/91 genotype are coexisting in Brazil (212). The only licensed live attenuated vaccine for IBV in Brazil is the Mass serotype and it was shown that Mass vaccine cannot protect birds against some of the Brazilian variants (43, 65).

5. Evolution of IBV

As noted from the emergence of new serotypes and variants, IBV is constantly evolving. RNA viruses, including IBV, exist as a large population with a diverse genetic pool created through rapid replication, which results in high mutation rates and recombination. Compared to the high fidelity of DNA polymerase, viral RNA dependent RNA polymerase (RdRp), utilized by RNA viruses, has a much high error rate and limited proof reading ability. Mutations including substitutions, insertions and deletions occur during the process of viral RNA replication (69). This broad genetic diversity facilitates the ability of viruses to adapt under various environmental pressures.

The average synonymous mutation rate of IBV is approximately 1.2×10^{-3} substitutions/site/year (102, 117). The presence of an exoribonuclease (ExoN) domain located in NSP14 has been identified for IBV and some other coronaviruses and is involved in proofreading

and repair (161, 195). It was shown that SARS-CoV and MHV-CoV with mutations in the ExoN domain exhibited impaired growth and increased mutation rates compared to wild type viruses (75, 76). These data confirmed that ExoN somewhat compensates the fidelity of the viral RdRp and helps the virus maintain its large genome size (63, 192).

Even though these mutations occur presumably throughout the entire viral genome, most mutations are found in the spike gene. Many mutations within functional genes associated with viral replication and assembly may generate deleterious mutants which eventually die out and do not contribute to the viral population (117). Emergence of new serotypes and variants are the result of mutations accumulating in the S gene (68, 144, 165). Generation of mutations in the S gene gives the virus advantages to evade the host immune system and to easily adapt to different hosts or tissues.

Recombination is another important mechanism involved in IBV evolution. It is believed that when two or more viruses infect the same cell RdRp can fall off of the original template strand and switch to a new template from a different virus. It is thought that the two viruses must share high sequence similarity in the crossover sites (45). This event can result in a change in pathogenicity of virus and emergence of new strains of IBV. The higher incidents of recombination occur in the S, nsp2, nsp3 and nsp16 genes (8, 117, 201). Recombination can potentially result in evolutionary advantages to virus subtypes by reducing mutational load and generating new virus populations. One documented example of emergence of a new virus by recombination is turkey coronavirus (TCoV) (116). TCoV is an enteric virus of turkeys and its genome outside of S shares high similarity (>86%) with IBV whereas the S protein shows less than 36% similarity with IBV (116). Presumably an unknown virus contributed to the

recombination event with IBV and giving rise to TCoV, which has altered pathogenicity and host specificity from IBV.

The extensive use of live attenuated vaccines to control IBV in the field can also provide more chances for positive selection and recombination. Immune pressure resulting from intense vaccination can lead to the emergence of new virulent IBVs such as the Georgia98 (GA98) strain, which was derived from DE072 (68, 144). In addition, persistence and circulation of vaccine viruses in vaccinated flocks can increase opportunities for the virus to mutate and to recombine with other viruses (206). Persistence of the Ark-DPI type vaccine virus provided an opportunity for mutations to occur resulting in the emergence of new Ark-like viruses (119, 120).

Part III.

Diagnosis

The clinical signs of IB are not specific and they are indistinguishable from those of several other viral pathogens, such as Newcastle disease virus, infectious laryngotracheitis virus and low pathogenic avian influenza virus, all of which cause upper respiratory disease in chickens. Due to the common clinical signs that are caused by various avian viruses, it is necessary to have specific diagnostic tools to identify these viruses when the clinical signs are present. Diagnosis of IB can be achieved by: 1) isolating virus, 2) detecting antigen, 3) detecting viral genetic material or 4) detecting a specific antibody response. In addition to the diagnosis of IBV, identification of the serotype or the genotype of the virus should be performed because IBV shows great antigenic variation and identification of circulating serotypes is necessary for proper protection.

1. Virus isolation

Since the primary target of IBV is the trachea, tracheal or choanal swabs or tracheal tissues can be used, especially within 3 to 5 days post infection, to obtain a high concentration of virus (59). Cloacal swabs or cecal tonsils collected post mortem are also valuable samples for virus isolation because virus spreads to nonrespiratory organs after initial replication in the upper respiratory tract (30). In addition, kidney, oviduct or enteric contents can be used, depending on the clinical history of the disease (30). Samples for virus isolation are prepared as suspensions containing antibiotics, filtered through 0.22um filters and inoculated into 9 to 11 day old SPF embryonated chicken eggs. Allantoic fluid collected 48-72 hours post-inoculation are subjected to further blind passages (30) and embryo lesions as well as death are analyzed. The characteristic lesions in chicken embryos include dwarfing, curling, ruffled feathers, hemorrhages, and kidney urinates. To observe characteristic lesions or embryo death, adaptation of IBV by several egg passages may be required. Thus, virus isolation can be expensive, labor-intensive and time consuming. Therefore a combination of virus isolation as a virus multiplication tool and a detection method for viral antigen or virus genome is more often used. In addition to embryonated eggs, tracheal organ cultures (TOC) obtained from 19 to 20 day old SPF chicken embryos can also be used for virus isolation (38). Tracheas are dissected into tracheal rings, which are placed in cell culture media with antibiotics. Ciliostasis caused by IBV can be observed after 48-72 hours of incubation, but may differ depending on the strain of IBV or amount of inoculation (37, 59).

2. Detection of viral antigen

IBV-specific antibodies, either in antisera or as monoclonal antibodies (Mabs), are used for IBV antigen detection (30). Antisera is collected from animals injected with IBV or certain viral proteins and may be a mixture of several antibodies against different IBV protein epitopes. Monoclonal antibodies (Mabs), designed to react to only a small number of epitope(s) on the IBV antigen, targets highly conserved areas.

Agar-gel precipitation test (AGPT)

The AGPT is a very simple immunological method used in the detection and identification of antigens and antibodies. Antibodies and antigens are placed in a series of wells in the gel and once diffusion of antibodies and antigens meet, specific antibody antigen reactions form an immunocomplex visible by a thin white precipitation line. The advantages of the AGPT are ease of use and low cost. In general it detects IgM so it can be used to diagnose infections early. However it is not very sensitive and for that reason it is rarely used. For antigen detection, several group specific antisera at various concentrations should be used because an imbalance in the antigen: antiserum ration can result in false negatives (148).

Immunofluorescent assay (IFA) and Immunoperoxidase assay (IPA)

The IFA and IPA can be used to detect IBV antigens in cells, both in culture and in tissues (59). The sensitivity and specificity of these assays rely on the use of polyclonal anti-IBV serum or specific monoclonal antibodies. Depending on the use of group-specific Mabs (or polyclonal antisera) or type-specific Mabs, the IFA can be either a group-specific or a type-specific test. For IFA, antibodies conjugated with a fluorochrome or enzymes are incubated with

fixed organ or tissues. The antibodies bind to IBV antigen directly and fluoresce under UV light. The amount of antigen in the sample must be large enough to be detected by staining, therefore, depending on the test materials, the sensitivity of the test may vary (59). For IPA, IBV-specific antibodies conjugated to the peroxidase enzyme are used to detect antigen in fixed organs or tissues. Observation of IPA test results does not require a fluorescence microscope and the staining of IPA is stable in contrast to IFA staining which is subject to photobleaching. The IPA can have non-specific IBV background staining, due to endogenous peroxidase present in the sample. Thus, this endogenous peroxidase activity has to be removed during the sample preparation (59).

Antigen-capture Enzyme-linked immunosorbent assay (ELISA)

Antigen-capture ELISA using monoclonal antibodies has been used to both detect IBV and differentiate between serotypes (18, 111, 221). Because of the large amount of virus antigen required for this assay, detecting IBV antigen directly from chicken organs by ELISA may result in false negatives. The confirmation of antigen in allantoic fluid of embryonated eggs or TOC can be performed using antigen-capture ELISA (59, 111, 168).

3. Detection of the IBV genome

Reverse transcriptase polymerase chain reaction (RT-PCR) has been used to detect IBV genomic RNA in a sample (30). The viral RNA genome in whole or in part is extracted from the sample and amplified by specifically designed forward and reverse primers. Most often, samples must be propagated in embryonated eggs to increase the viral load and achieve better sensitivity (59). Generally, the S1 portion of the spike protein gene is targeted for amplification. After amplification, additional steps such as sequencing of the RT-PCR product, restriction enzyme

fragment length polymorphism (RFLP) (139, 146, 197) or hybridization of IBV specific probes (123, 138, 230) are performed for differentiating serotypes or variants. Conserved or variable regions in the IBV S1 gene have been noted and utilized to serotype IBV isolates by molecular methods. (124, 135, 166)

4. Detection of antibodies

Most ELISA tests are group specific and therefore do not differentiate serotypes. Broadly used ELISA plates for IBV are coated with whole IBV antigen and detect IgG in serum. The ELISA test can be used for detection and titration of antibodies. But the antibody level does not always correlate with the protection level (23). Also the antibodies induced by vaccination cannot be differentiated from infection-induced antibodies.

The virus neutralization (VN) assay and hemagglutination inhibition (HI) test can be used for detection of type-specific antibodies, although cross-reaction between serotypes exists (30). The VN assay can be carried out in cell cultures or embryonated eggs. This assay requires sera that only reacts with designated serotypes with no cross reaction occurring with others. For the HI test, IBV is treated with neuraminidase enzyme before testing because IBV is not naturally hemagglutinating. Hemagglutination-inhibiting antibodies are induced against the spike protein. The HI test can be used to detect serotype–specific antibodies after a single infection or inoculation but a second or subsequent infection with heterologous IBV can lower the specificity due to generation of cross reactive antibodies (209).

5. Strain Classification

Typing of IBV strains in the field is the essential first step in choosing the right vaccine type for control. Typing IBV is also useful for research purposes, namely to study viral evolution and epidemiology and for disease surveillance. Classification of IBV strains has been hindered due to the nature of IBV, characterized by its high mutation rate and lack of standardization of tests and nomenclatures (59). Recombination events between different types of IBV or mutations in amino acids can alter the viral serotypes or genotypes (27, 127, 136, 144). Therefore IBV strains can be the result of multiple cross-reactions and clear classification of strains may be difficult. The strain of IBV can be classified by functional or non-functional groups. Functional groups are determined by the biological function of a virus and result in serotypes and protectotypes. Non-functional groups are determined by examination of the viral genome.

Serotype

Serotyping of IBV mainly relies on features of the S1 protein of IBV because it contains virus neutralizing and serotype specific epitopes. The VN or HI tests are commonly used for serotyping of IBV. The VN test is based on the reaction of the IBV strain and IBV-serotype specific antibodies obtained from chickens. Embryonated eggs (50, 57), TOC (39, 64, 130) and chicken kidney cells (49, 214) have been used in VN tests. The β-method VN test, which uses a constant virus titer with varying dilution of antibodies, is preferred over the α-method that uses varying dilutions of virus with fixed concentration of antibodies (59). The HI test detects serum antibodies capable of preventing agglutination of chicken red blood cells (RBSs). Though serotyping results obtained by HI can be highly strain specific, high and variable cross-reactions are often observed (19) and the differences between the strains are less clear than when the VN test is used (40, 59).

Protectotype

Protectotypes of IBV can provide direct information that is practically applicable to designing a vaccine program for IBV (59). Cross-protection between two strains defines them as belonging to the same protectotype. Distinct serotypes of IBV can provide cross-protection against each other and be grouped together in one protectotype. Different serotypes can share common epitopes which contribute to cross-immunity (26, 59). The number of vaccine serotypes used in a vaccination strategy can be reduced by using the protectotype classification (59).

Genotype

IBV strains can be classified based on genetic characterization of the S1 region, especially the S1 hypervariable region (HVR). Genotyping methods include sequencing, RT-PCR amplified regions of genotype specific areas, or restriction fragments length polymorphism (RFLP), which measures the position of enzyme cleavage sites. There have been several studies on correlation between genotype and serotype of IBV strains (135, 139, 146), however, careful consideration is needed before direct application of genotype in the field.

6. Microsphere-based assay

Microsphere-based suspension array, or Luminex xMAP system™ is a high throughput analyte detection system with flexible application (73). Polystyrene or magnetic microspheres that are internally dyed with two distinct fluorochromes, and each microsphere set represents a single spectral address that can be combined with up to 500 different microsphere sets in a single reaction. Upon direct DNA hybridization of a PCR amplified DNA product with target specific oligonucleotide probes bound to the microsphere, signals from microspheres and reporter dye are detected by two lasers of the Luminex system. The red diode laser excites the two internal dyes

in the microsphere and yttrium aluminum garnet (YAG) laser excites the reporter dye bound to the microsphere surface (73). A brief assay procedure is as follows: first, capture probes are designed. Probes are modified with 5' amino-C6 linkers for covalent linkage to carboxylated microspheres. Viral RNA is extracted from the sample and amplified by RT-PCR and a 5' modified forward primer is used to label the DNA amplicons with a reporter dye. Then DNA amplicons are hybridized with the microsphere-bound probes and an incubation step follows. After washing the unbound DNA, the Luminex system reads excitement of the dyes and shows the results as a median fluorescent level (MFI).

Microsphere-based assays have been successfully used for the diagnosis of many infectious pathogens, including simultaneous detection of multiple bacteria such as bacterial vaginosis associated bacteria (72), *Salmonella* (74), *Shigella* (36), *E.coli* (36), *Listeria monocytogenes*, *Camplybacter jejuni*, *Staphylococcus aureus* (74, 85), respiratory associated adenovirus serotypes (216), human papillomavirus (58), avian influenza (31, 137), human respiratory disease associated viruses (152), foot-and-mouse disease virus (FMDV) (104, 175), *Aspergillus* spp. (79, 80), clinically relevant fungal pathogens (141), *Trichosporon* spp (66).,and *Candida* spp. (54). This microsphere-based assay can be successfully adapted as a diagnostic tool for IBV detection and differentiation of IBV serotypes by utilizing specific probe hybridization.

Part IV.

Vaccination & Current challenges on vaccination

1. Control & Vaccination of IBV

As IBV is highly transmissible via aerosol and direct contact in susceptible birds (60) and is able to persist in the respiratory tract and feces for several weeks (1, 133). The best prevention method for IBV is reducing the naïve birds in a flock by having only one-age bird on the farm, as well as proper cleaning and disinfecting and repopulating with one-day old chicks. However in current production systems with multiple ages, in a house with high density rearing, it is difficult to control IBV through management. Consequently vaccination against IBV to prevent production loss is necessary. Both live attenuated and killed vaccines are widely used for vaccination.

Live attenuated vaccine

Live attenuated vaccines are used in meat-type chickens (broilers) and for initial vaccination of layers and breeders for priming purposes. In common practice, broilers are vaccinated with live vaccines at one-day of age in the hatchery and boosted with live vaccines of the same or different serotypes in the field at two weeks of age. In layers and breeders, live vaccines are used at two or three weeks of age to avoid potential inhibition by maternal antibodies in chicks, followed by more live vaccination until onset of egg production (30). A few weeks before egg production killed vaccines are used to induce serum antibody production in layers and breeders.

Live vaccines are prepared by attenuating viruses through serial passages in embryonated chicken eggs until the virus is no longer pathogenic to birds but still maintains its immunogenicity. The degree and stability of attenuation varies among vaccine strains.

The efficacy of live vaccine is affected by many factors including age of the chick, maternal antibody level, vaccine application method, immunocompetency of the host, vaccine schedule, selection of vaccine strains, immunogenecity of selected vaccines and genetic differences of chickens. In well-vaccinated birds, a certain serotype or genotype vaccine provides adequate protection against homologous challenge and may provide partial protection against other strains of IBV. Under optimal conditions, vaccinated chickens may have immunity up to several months (11), although immunity induced by IBV vaccine is normally short live and starts to decline by 6 to 12 weeks after vaccination (53, 98). In areas with potential of IBV outbreaks, broilers, which are processed by six weeks of age, may be revaccinated and layers and breeders, which are kept longer (a year or more), are periodically vaccinated with more than one strain of live vaccine during the laying period to maintain protection.

To obtain a broad range of protection when using live vaccines, multiple serotypes can be used. Using two different vaccine serotypes, applied with a 2-week interval rather than combined on the same day, provided broader cross-protection against different IBV types then using a single vaccine type (42, 200). It also has been shown that a bivalent vaccine with Mass and Ark strains protected birds with a higher level of cross-protection against certain heterologous challenge strains than other vaccine combinations (92-94). But Ladman and his colleagues also showed the Mass and Ark vaccine did not provide significant protection against a nephropathogenic strain of IBV (140).

The Massachusetts serotype is the most widely used vaccine strain and is the only available option for IBV vaccination in some countries. In Europe, different forms of the Mass serotype including H120, Ma5 and Modified Mass (MM) are given to birds (132) singly or in combination with other IBV serotypes such as Holland (D274, D1466, and 4/91) (132). In the U.S, the Massachusetts, Arkansas and Connecticut serotypes are widely used in addition to GA98, and De072, which are more regionally used (119).

Experimental application methods of a live vaccine are eye drop, intranasal and intratracheal inoculation. Commercial vaccine administration is performed by coarse spray, aerosol, or drinking water. In the hatchery, vaccines are applied to chicks using a spray cabinet while in the field, boost vaccination using aerosol spray or drinking water is performed. Whilst mass applications are selected due to convenience and economic reasons, failure in uniformity of vaccine administration can occur (61, 120) and lead to prolonged circulation of the vaccine virus in the flock and increase the possibility of vaccine virus regaining virulence, which often described as “rolling reaction” (109). Vaccines against IBV can be easily inactivated by inappropriate vaccine storage, handling and application, which may result in decreased vaccine efficacy in the field (61, 95, 120). Therefore, careful attention is required for proper vaccine application.

Inactivated vaccine

Inactivated vaccines for infectious bronchitis viruses were developed in the 1960s and 1970s (24, 46, 105). Inactivated vaccine is commonly used in layers and breeders a few weeks before the onset of egg production. Inactivated vaccines are prepared with virus inactivated by addition of formalin or other inactivant and combined with an adjuvant such as mineral oil (126). Individual subcutaneous or intramuscular injections are required for inactivated vaccine

application. To obtain a good level of protective immunity using inactivated vaccines, proper priming by live vaccines is necessary. Single application of inactivated vaccine does not provide proper protection against egg loss and ciliostasis (15, 52, 154, 167). The main purpose of inactivated vaccine is to protect hens from production egg drops (154) by increasing the level of antibodies in serum and to provide passive maternal antibodies to chicks. Inactivated vaccines are not as effective as live vaccines to protect birds from respiratory infections. Since inactivated vaccine viruses do not replicate in birds, mucosal cellular immunity or IgA production is not induced.

Subunit vaccine & recombinant vaccine

Subunit vaccines using S1 spike subunit protein purified from virus (28, 112) and baculovirus expression systems (197) has been studied in experimental settings. Commercial application was unsuccessful because it requires multiple inoculations to obtain protective immune responses and the percentage of birds acquiring protective immunity was below 50%.

Fowl pox virus (215) and fowl adenovirus (129) have been used as a vector system for expressing and delivering IBV S1 protein. In an experimental setting, fowl adenovirus vector expressing S1 protein successfully induced protection in chickens only after a single application against a homologous or heterologous strain challenge virus (129). The demonstration of successful vaccination using recombinant vaccine opens the possibility of alternative vaccination against IBV.

2. Current challenge in Ark type vaccine in the U.S

Since the Ark-DPI strain was first identified in Delmarva broiler chickens and attenuated for use as a live vaccine (83, 88, 91), it has been used as a vaccine in the U.S. The Ark-DPI virus

was attenuated after more than 100 passages in embryonated chicken eggs and its virulence and immunogenicity in chickens was tested (88). This attenuated seed virus was then further attenuated to be used as live vaccine. Despite extensive Ark-DPI vaccine usage along with other IBV serotypes in the Southeastern U.S, Ark type IBV has been frequently isolated from IBV outbreaks (119, 172, 204). Ark-DPI was the most prevalent IBV type isolated from clinical samples obtained over a period of 11 years (1994-2004), ranging from 23-65% of isolations each year. In addition, Ark like isolates continued to emerge during this period, which indicates genetic drift of persisting Ark-DPI vaccine viruses in the field (119). Nix and his colleagues (172) also indicated Ark type as the most prevalent serotype of IBV during 1993-1997 and further identified Ark-like subtype viruses by antigenic comparison and S1 gene analysis. A similar result showing predominance of Ark type was obtained from analysis of IB clinical samples submitted to Alabama state diagnostic laboratories (204). Predominance of Ark type IBV in various poultry producing areas was also mentioned in a report from the American Association of Avian Pathologists (AAAP) Respiratory Diseases Committee in 2009 (193).

The factors related to the persistence of Ark type viruses in the field are yet to be discovered. There was a concern of breaking through the immunity acquired from commercial Ark-DPI vaccine due to genetic/antigenic drift of Ark subtypes isolated from the field. However Sander and colleagues (182) proposed that poor protection of Ark-DPI vaccines in the field is due to lack of proper immunization rather than antigenic drift of Ark viruses. The inappropriate priming of vaccinated birds and consequent poor protection against Ark challenge viruses was confirmed by Jackwood et al., (120). In that study it was also shown that the Ark-DPI vaccine viruses were the only isolated IBV type from vaccinated birds and no other serotypes used in the study were isolated from birds, indicating the persistance of Ark vaccine viruses in vaccinated

birds. Another experimental study on isolation of Ark and Mass vaccine viruses found that Ark vaccines were detected later than Mass vaccine (3) which confirmed the persistence of Ark vaccine in birds.

Since subpopulations within the Ark serotype were first discovered (119, 172), other researchers have found high genetic variability of commercial Ark type vaccines. Spike gene sequences from Ark virus populations obtained from commercial Ark vaccines were analyzed and the existence of various degrees of genetic heterogeneity, especially in the S1 subunit, were found, suggesting that distinct Ark virus populations were selected differently during commercial vaccine production (155, 206). These subpopulations in vaccines were selected as early as 3 days post vaccination. McKinley et al., (155) showed that, in addition to existing subpopulations, there were also mutations and deletions in the S gene involved in the *in-vivo* selection process. Collectively, Ark vaccines show high genetic variability and subpopulations in vaccines undergo selection *in-vivo*. It is possible that the genetic heterogeneity of Ark virus gives an advantage for some subpopulations persisting longer in vaccinated birds. However, this needs to be further studied to understand the Ark type virus persistence in the field.

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CHAPTER 3

SIMULTANEOUS DETECTION OF FIVE MAJOR SEROTYPES OF INFECTIOUS BRONCHITIS VIRUS BY A MULTIPLEXED MICROSPHERE-BASED ASSAY[1]

[1]Roh, H-J, D.A.Hilt, and M.W.Jackwood. Submitted to *Journal of Veterinary Diagnostic Investigation*, 03/19/13.

Abstract

Infectious bronchitis virus (IBV) is an avian coronavirus with major economic importance to commercial chicken producers worldwide. Due to the existence of multiple serotypes and variants of the virus that do not cross protect, it is important to diagnose circulating serotypes and choose the right vaccine type for successful protection. In an effort to improve conventional diagnostic tests, a microsphere-based assay was developed and evaluated for simultaneous detection of the most common IBV vaccine serotypes in the USA; Arkansas (Ark), Connecticut (Conn), Massachusetts (Mass), Delaware (DE072), and Georgia 98 (GA98). The analytical specificity and sensitivity and diagnostic specificity and sensitivity were evaluated. The microsphere-based assay was highly specific to designated serotypes, and generated reproducible data. Comparing the microsphere-based assay to nucleotide sequencing, the two methods agreed more than 93% (Kappa value >0.77). In addition, the microsphere-based assay could detect co-infections in clinical samples. These results demonstrate the utility of the microsphere-based assay as a rapid and accurate diagnostic tool with the potential for high throughput diagnosis.

Key words: Arkansas (Ark); Bio-plex; Connecticut (Conn); Delaware 072 (DE072); diagnosis; Georgia 98 (GA98); Infectious bronchitis virus (IBV); Luminex; Massachusetts (Mass); microsphere-based assay; multiplexed; serotyping.

Introduction

Infectious bronchitis virus (IBV) is a positive sense single-stranded RNA virus with a genome ranging from 27 to 28Kb. IBV belongs to the group gamma coronavirus, in the family *Coronaviridae.* The virus is highly contagious and primarily infects epithelial cells in the upper respiratory tract of chickens, but can also infect epithelial cells in the kidney, oviduct, testes, and alimentary tract (2). This economically important disease affects chickens, which are the primary host but pheasants (Phasianus colchicus), and peafowl (Galliformes) have also been infected. The virus causes an upper respiratory disease in young chickens and can cause decreased egg production and egg quality in hens. Some strains of IBV target the kidneys causing an interstitial nephritis. Since the first identification of IBV in 1930s in the USA (17), many serotypes and variants have been identified worldwide. Different serotypes do not confer cross-protection (38), which makes it extremely difficult to control the disease (3). Antigenic variation of IBV is largely due to mutations and recombination that affect the spike protein. Spike proteins are made up of two subunits (S1 and S2) and form club-shaped projections that extend from the surface of the virus particle. The S1 subunit, which makes up the terminus of the spike, contains epitopes that induce neutralizing antibodies. Mutations in the S1 gene that change the epitopes can result in virus particles escaping the immune response (3). Currently, vaccination with attenuated live virus is the best strategy for control of IBV. Because of the poor cross-protection between heterologous serotypes, diagnosing circulating viruses in the field and choosing antigenically homologous vaccine strains are critical steps for the successful control of IBV.

Conventional diagnostic methods to differentiate IBV serotypes include virus isolation in specific pathogen free (SPF) embryonated eggs followed by virus neutralization (VN) tests, hemmaglutination inhibition (HI) tests, or antigen-capture enzyme linked immunosorbent assay

(ELISA) using monoclonal antibodies (1, 20, 39). However, genetic based tests to identify IBV types have become the test of choice since the discovery that sequences in the S1 gene are correlated with different serotypes of IBV (23, 25, 34). Reverse transcriptase polymerase chain reaction (RT-PCR), targeting the S1 portion of the spike protein, followed by sequencing of the RT-PCR product (32), restriction enzyme fragment length polymorphism (RFLP) (29, 31) or hybridization with IBV specific probes (22, 24, 28) have been developed for differentiating serotypes and variants of the virus.

Microsphere-based suspension arrays[a] are a relatively new diagnostic platform that enables high throughput detection of nucleic acids as well as other analytes. Microspheres contain two internal fluorochromes with different intensities giving each microsphere a unique spectral character. This unique spectral character theoretically allows up to 500 different microspheres to be combined and used in the microsphere-based assay (13). The test involves direct hybridization between PCR amplified DNA products from clinical samples and target specific oligonucleotide probes coupled to the microspheres. The amplified products are conjugated with reporter dyes at the 5' or 3' ends. A microsphere analyzer uses lasers to excite the internal dyes of the microsphere and the reporter dye conjugated to PCR products, and reads the fluorescent levels. The result is reported as a median fluorescent level (MFI) identifying the microsphere spectral address and the presence of the PCR amplified product. Microsphere-based assays have been used for the diagnosis of many infectious pathogens such as bacterial vaginosis associated bacteria (12), *Salmonella (14), Shigella (7), E.coli (7), Listeria monocytogenes, Camplybacter jejuni, Staphylococcus aureus* (14, 18), respiratory associated adenovirus serotypes (37), human papillomavirus (9), avian influenza (6, 26), human respiratory disease

associated viruses (33), foot-and-mouse disease virus (FMDV) (19, 35), *Aspergillus* spp.(15, 16), clinically relevant fungal pathogens (30), *Trichosporon* spp(11).,and *Candida* spp.(8).

The majority of IB viruses isolated from commercial chickens are vaccine type viruses and rapidly distinguishing them from each other and from variant viruses is critical for control of IB. In this study, we developed and evaluated a multiplexed microsphere-based assay for typing the five major IBV vaccine viruses used in the USA. The analytical sensitivity and specificity of the multiplexed microsphere-based assay was analyzed, and evaluation of the assay as a potential diagnostic tool for IBV was performed using previously identified clinical samples. The results were compared to current tests utilizing RT-PCR amplification and nucleotide sequencing.

Materials and Methods

Virus samples

For initial assay development and optimization, previously identified virus stocks were used. Arkansas Ark/ArkDPI/81 (Ark-DPI), Massachusetts Mass/Mass41/41 (Mass41), Connecticut Conn/Conn46/51 (Conn), Delaware DE/DE072/92 (DE 072), and Georgia 98 GA/GA98/0470/98 (GA98) were propagated in 9-10 day-old specific pathogen-free (SPF) embryonated chicken eggs and the 50% embryo infectious dose (EID_{50}) titer was calculated by the Reed and Muench method. Other chicken respiratory viruses, which include Newcastle disease virus (NDV), infectious laryngotracheitis virus (ILTV) and avian influenza viruses (AIV), as well as *Mycoplasma gallisepticum* (MG) used in this study were obtained from the Poultry Diagnostic and Research Center (PDRC), Athens GA.

Design of primers and serotype specific probes

Primers and probes were designed against a hypervariable region in the IBV S1 gene for the Ark-DPI, Mass41 and Conn viruses. Since DE072 and GA98 are antigenically related and show high sequence similarity in the S1 gene, a single probe was designed to detect both serotypes. Currently available sequences of IBV viruses in GenBank were used and aligned using the ClustalW method in DNASTAR[b] and regions specific to each serotype were identified and used to develop serotype specific probes. Forward and reverse RT-PCR primers were designed in conserved areas flanking the probes. The amplified product is approximately 537bp in length. Basic Local Alignment Search Tool (BLAST) analysis was performed (blast.ncbi.nlm.nih.gov) to verify the specificity of probes and primers. The primers and probes were synthesized by a commercial company[c]. The serotype specific probes contain an amino-modified 6-carbon spacer at the 5' end for binding to the beads. The forward RT-PCR primer included a 5'-biotynilation modification for binding to the reporter dye. In addition, biotinylated oligonucleotides (antiprobes) complementary to the probes bound to the microspheres were obtained to evaluate binding of the probes to the microspheres.

RNA Extraction and RT-PCR amplification

Viral RNA was extracted from 200µl of allantoic fluid or clinical samples according to the manufacturer's instructions[d] and eluted into 50µl of buffer. Extracted RNA was stored at -80°C. The RT-PCR reaction was performed using a commercially available RT-PCR mix following the manufacturer's instructions[e]. The RT-PCR reaction mixture included 10µl 5X RT-PCR reaction buffer, 10mM of each dNTP, 12.5µM of each primer, 40U RNase inhibitor[f], 2µl DTT, 2µl $MgCl_2$, 1µl of Enzyme mix, and 5µl of extracted RNA. The RT-PCR reaction was performed on a thermocycler[g] using the following conditions: one cycle of 42°C for 60min and

95°C for 5min; 10 cycles of 94°C for 30sec, 53°C for 30sec, and 68°C for 90sec; 25 cycles of 94°C for 30sec, 53°C for 30sec, and 68°C for 90sec adding 5 sec per cycle, and final extension at 68°C for 7min. For the limit of detection test, RT-PCR products were agarose-gel purified using a commercially available kit according to the manufacturer's instructions[h]. For other tests including specificity and clinical sample evaluation, RT-PCR products were used directly without agarose-gel purification.

Microsphere-based assay

Coupling of serotype specific probes and polystyrene microspheres was performed according to the manufacturer's bead coupling protocol[i]. We added 10µl of biotinylated RT-PCR product directly to 33µl of working microsphere mixture (single or multiple microspheres) in 7µl of Tris-EDTA (TE) buffer (pH 8.0) in a single tube. Hybridization was performed at 95°C for 5 min followed by 55°C for 15 min. After hybridization, the mixtures were centrifuged at 2,250 X g for 3 min and the pellet was resuspended in 1X tetramethylammonium chloride (TMAC) containing 3µl/ml of streptavidin-R-phycoerythrin[j] followed by a 5 min dark incubation. The beads hybridized with amplified RT-PCR product were analyzed using the microsphere reader[k] and the signal was expressed as mean fluorescence intensity (MFI). The MFI signals greater than triple the highest background MFI for a given microsphere set was considered positive. Multiple positive (Ark-DPI, Mass41, Conn, DE072 and GA98 positive controls) and negative controls (bead control, RT-PCR control) were included in each run.

Specificity and limit of detection of the microsphere-based assay

To determine specificity of the microsphere-based assay, singleplex and multiplex assays were performed in triplicate with RNA extracted from known positive allantoic fluid samples of different IBV serotypes as well as with nucleic acid extracted from samples containing other

avian respiratory pathogens, including AIV, NDV, ILTV, and MG. Extracted RNA from samples was processed as described above.

The limit of detection for each given microsphere set was determined using 2-fold serial dilutions of gel-purified biotinylated RT-PCR products. Purified DNA concentrations were determined using a spectrophotometer[l]. Singleplex and multiplex microsphere-based assays were performed in triplicate and the limit of detection was determined by the lowest dilution giving positive MFI.

Evaluation of the microsphere assay

For evaluation of the assay, 59 clinical samples collected from chickens were obtained from Dr. Holly S. Sellers (PDRC, Athens, GA). Clinical samples were previously identified by RT-PCR followed by nucleotide sequencing. The multiplexed microsphere-based assay was performed using RNA extracted from clinical samples and test results were compared to the sequencing results.

Restriction Fragment Length Polymorphism (RFLP) analysis

RNA extracted from clinical samples was used to amplify the entire S1 gene region by RT-PCR using previously published primers (23, 31) (NEWS1OLIGO5':5'-TGAAAACTGAACAAAAGAC-3', Degenerate3':5'-CCATAAGTAACATAAGGRCRA-3'). The RT-PCR conditions were previously described(29). The S1 gene RT-PCR products were gel purified using a commercially available kit[h] and digested using restriction endonucleases (*Bst*YI, *Hae*III, and *Xcm*I) according to the manufacturer's recommendation[m]. Digested samples were electrophoresed on 2% agarose gels and the restriction fragment length patterns of the samples were analyzed and compared to the reference viruses.

Results

Confirmation of bead coupling and selection of serotype specific probes

The coupling of probes to each set of microspheres was evaluated using biotinylated oligonucleotides (antiprobes) complementary to the probes. The antiprobe was serially diluted (5fmol ~ 200fmol) and the MFI of each reaction was compared. The MFI of antiprobe increased linearly with the quantity of antiprobe in the sample (data not shown). To verify the specificity of the probes for the designated IBV serotypes, singleplexed assays were performed, in triplicate, using the amplicons generated from each reference virus. Data for the probes that only detected the targeted IBV serotypes with no cross-reactivity are shown in Fig.3-1A. The probes were designated as Ark-P, Mass-P, Conn-P, and Del/GA98-P, with the Del/GA98-P designed to detect both DE072 and GA98 serotypes. Background fluorescence was determined using negative controls, which were included in all assays.

Specificity of the multiplexed microsphere-based assay

The specificity of the multiplexed microsphere-based assay was examined by testing 17 different pathogens, including different serotypes of IBV and the data are shown in Table 3-2. As shown in Fig.3-1 and Table 3-2, the assay was able to detect the target IBV serotypes, whereas non-specific binding to other IBV serotypes and pathogens was not detected.

Limit of detection of the microsphere-based assay

The limit of detection of the singleplexed and the multiplexed assay for biotinylated amplicons generated using RT-PCR was estimated by analyzing 2-fold dilutions of amplified S1 gene products from Ark-DPI, Mass41, Conn, DE072, and GA98, and the limits of detection of the singleplexed assay were 6.4ng, 3.75ng, 7.5ng, 15.9ng, and 7.2 ng, respectively (Table 3-3). The minimum amounts of DNA detected for the multiplexed assay were 9.05ng, 4.2ng, 10.5ng,

15.75ng, and 7.2ng respectively. Corresponding DNA amplicon copy numbers were calculated based on the assumption of the average weight of a base pair (bp) is 650 Daltons. The equation is [Number of copies = (DNA amount (ng) * 6.022 × 10^{23}) / (length of template (bp) * 1×10^{9} * 650)] and data are shown in Table 3-3.

Reproducibility of the multiplexed microsphere-based assay

To confirm the reproducibility of the multiplexed microsphere-based assay, intra-assay (the individual test results within a single run) and inter-assay (the individual test results from one run to another) variability was evaluated. For each probe with each targeted reference virus, the coefficient of variation (CV) of MFI values within a single run (intra-assay) ranged from 0.03 to 0.05, and that of inter-assay ranged from 0.01 to 0.08 (data not shown).

Evaluation of clinical samples

To evaluate the performance of the assay as a diagnostic tool, 59 clinical samples were tested without prior knowledge of their type using the multiplexed microsphere-based assay. Clinical samples consisting of tracheal swabs were collected from chickens and nucleotide sequencing was used to identify the IBV type in each sample. Data from the microsphere-based assay and nucleotide sequencing are compared in Table 3-4. In addition, seven samples were identified as co-infections of two different serotypes of IBV by the multiplexed microsphere-based assay (Table 3-5). To verify that the samples indeed contained two different IBV types, we conducted RFLP analysis on the amplified S1 gene and found that both Ark-DPI and Mass41 type viruses were present in clinical sample #82323, which was only identified as Ark virus positive by nucleotide sequencing. In addition, sample #82427 was also determined to be a co-infection of Mass41 and DE072/GA98 type viruses by RFLP analysis (data not shown). The other samples where a weak MFI signal was observed could not be confirmed as containing 2

IBV types by RFLP analysis. Based on the 59 clinical samples tested, the specificities of detection for the different serotypes of IBV were 88.8% (Ark-P), 93.2% (Mass-P), 100% (Conn-P), and 96.6% (Del/GA98-P), and the sensitivities were 100% for all targeted serotypes. Agreement between the multiplexed microsphere-based assay and the nucleotide sequencing results for all tested viruses was >93% (Kappa correlation > 0.77) (Table 3).

Conventional verses microsphere-based assays

In Fig.3-2 we compare the procedures and timelines for conventional IBV serotype identification assays and for the microsphere-based assay. The VN test, a gold standard for serotyping IBV, takes an average of 7 days from incubation to data analysis. Extraction of RNA and reverse transcription steps are common to nucleotide sequencing and microsphere-based assays. Nucleotide sequencing requires approximately one to two additional days to acquire sequencing data, whereas the microsphere-based assay takes less than one hour, including sample preparation time, to finish data analysis.

Discussion

IBV is one of the most important pathogens in chickens causing a significantly negative economical impact on the poultry industry worldwide. Infection with IBV can be moderated by vaccination, but due to its numerous serotypes and variants that do not cross protect, constant surveillance of circulating viruses are needed so the appropriate vaccine can be selected. The VN test, is the definitive serotype identification test but an increasing number of IBV variants make it almost impossible to conduct the VN test for all possible serotypes (10). The alternatives to traditional serotyping tests are molecular methods that identify virus genotype such as RFLP analysis and nucleotide sequencing the RT-PCR amplified S1 gene.

In this study, we developed a multiplexed microsphere-based assay to identify four major IBV serotypes commonly used as vaccines. Distinctive serotypes of IBV are associated with differences in the sequence of the S1 glycoprotein, and unique hypervariable regions have been previously identified (4, 27) and correlated with different types of the virus (5, 25, 36). The microsphere assay described in this study also targets the S1 hypervariable region. Universal primers were designed based on conserved sequences, and serotype specific probes were designed to anneal to the hypervariable regions allowing us to identify 4 different genetic types (Ark-DPI, Mass41, Conn and DE072/GA98). The DE072 and GA98 virus types share high antigenic similarity, provide significant cross-protection (21) and have limited variability in the S1 hypervariable region. This limited variability prevented us from designing unique probes for each virus.

The IBV type specific probes were tested against reference viruses to ensure no cross reactivity occurred with non-targeted IBV serotypes as well as other selected avian upper-respiratory tract pathogens. The analytical sensitivity of the probes for the reference viruses in singleplexed and multiplexed assays ranged from 3.75ng to 15.9ng, corresponding to 6.46×10^9 to 2.74×10^{10} genome copy numbers. Under multiplexed conditions, the sensitivity of the probe was slightly lower.

We examined 59 clinical samples and found no statistically significant differences between the microsphere-based assay and nucleotide sequencing (>93.2% agreement for all of the probes). The sensitivity of each probe against its target IBV type was 100%, and the specificity was >89.1%. The kappa correlation values between two methods were higher than 0.77, which indicates the microsphere-based assay is as specific as the nucleotide sequencing method.

One unexpected finding from our analysis of clinical samples was that two different IBV types were identified by the microsphere assay in samples previously identified by sequencing as having only one IBV type. Typically when more than one IBV type is present in a sample, the sequencing data is not readable. However, nucleotide sequencing detected Ark-DPI for clinical sample 82323 and Mass41 for clinical sample 82427, whereas the microsphere-based assay detected Ark-DPI and Mass41 for clinical sample 82323 and Mass41 and DE072 for clinical sample 82427. We used RT-PCR amplification of the S1 gene followed by RFLP analysis to confirm these results. Typically multivalent IBV vaccines are given to commercial poultry to provide a broad immune response. Thus, the presence of more than one IBV type in a clinical sample is not uncommon. Detecting multiple IBV types in a single sample is a distinct advantage of the microsphere-based assay over nucleotide sequencing.

Three of the clinical samples (67297, 67634, 83188) previously identified as the GA08 IBV type by nucleotide sequencing were expected to be negative in the microsphere-based assay, but instead all three were weakly positive for Ark-DPI and Mass41. When we tried to confirm the presence of Ark-DPI and Mass41 in those samples using the RFLP test, we only detected the GA08 type of IBV. Our tests with the Ark-P and Mass-P probes showed that they did not cross-react with the GA08 virus (Table 3-2.) so it is possible that a low level of Ark-DPI and Mass41 could indeed be present but undetectable in those samples by sequencing or RFLP analysis. It is interesting to note that those clinical samples were obtained from chickens previously given live attenuated Ark-DPI and Mass41 vaccines. However, it is also possible that the weak positive signals obtained with the microsphere-based assay, could be false positive results.

We found that the microsphere-base assay requires less than 5 hours from start to finish, providing same-day results. In addition, procedures such as RNA extraction and washing

between hybridization and incubation steps can easily be automated to further reduce handling time and manipulation errors. In addition to automation, adaptability to high throughput format as well as increased flexibility by introducing a 96-well plate format and more probes is possible. This is all in addition to the most significant advantage of the test, which is multiplexing to identify more than one IBV type in a single sample.

In conclusion, we have developed and evaluated a multiplexed microsphere-based assay targeting the hypervariable S1 gene region to identify commonly used IBV vaccine serotypes. The assay is comparatively rapid, specific, and correlates well with conventional identification methods with the advantage that it can detect more than one IBV type in a sample. In addition, the availability of microspheres with different spectral addresses makes it possible to extend the test to include more IBV types. Thus, it appears that this multiplexed microsphere-based assay for IBV shows good potential as a research and a diagnostic tool.

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Sources and manufacturers

a. Luminex xMAP system^{TM}, Luminex, Austin, TX.

b. Lassergene8, Madison, WI.

c. Integrated DNA Technologies., Novato, CA.

d. High Pure RNA isolation kit, Roche Applied Science, Indianapolis, IN.

e. Titan One Tube RT-PCR System, Roche Diagnostics GmbH, Mannheim, Germany.

f. Recombinant RNase Inhibitor(5000 U), Takara Bio, Inc., Japan.

g. DNA Engine Peltier Thermocycler, Bio-Rad Laboratories, Inc., Hercules, CA.

h. QIAquick gel extraction kit, Quiagen, Valencia, CA.

i. Bio-Plex bead coupling protocol. Bio-Rad Laboratories, Hercules, CA

j. Invitrogen Molecular Probes, Eugene, OR

k. Bioplex microarray reader, Bio-Rad, Hercules, CA

l. Nano drop spectrophotometer, Nanodrop Technologies, Inc., Wilmington, DE

m. New England Biolabs, Beverly, MA

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Table 3-1. Primers and probes used in this study.

Primer/Probe	Target	Nucleotide sequence (5' → 3')	5' Modification
IBV-F'-Bio IBV-R'-537	Universal	5'-TAGTCACYMTTTTGTKTGCACTA*-3' 5'-TTAGANGTRWAAASRAGRTYRCCATTTAA-3'	Biotin
Ark-P	Arkansas	5'-CACAAAAGATTCGTTGTCATATAAATT-3'	NH_2-C_6'
Mass-P	Massachusetts	5'-AGGTGAAGAGCCTGCATTATTAGATTC-3'	NH_2-C_6'
Conn-P	Connecticut	5'-ACCAATAATACCAACAATACACTCTCTTAA-3'	NH_2-C_6'
Del/GA98-P	Delaware 072 and GA98	5'- ACTATGCAAYTATGACCRGTTCCACCAC-3'	NH_2-C_6'

*Degenerate primer abbreviations are as follows: Y, C or T; M, C or A; K, T or G; N, all; R, A or G; W, A or T; S, G or C;

Table 3-2. Analytic specificity of multiplexed microsphere-based assay against pathogens used in this study.

Virus	Subtype/serotype	Multiplexed probe specificity			
		Ark-P	Mass-P	Conn-P	Del/GA98-P
IBV*	Ark-DPI	+	–	–	–
	Mass41	–	+	–	–
	Conn	–	–	+	–
	DE072	–	–	–	+
	GA98	–	–	–	+
	GA08	–	–	–	–
	Iowa	–	–	–	–
	Florida	–	–	–	–
	JMK	–	–	–	–
	Gray	–	–	–	–
	Holte	–	–	–	–
	Ca99	–	–	–	–
NDV	Lasota type	–	–	–	–
ILTV	USDA†	–	–	–	–
AIV	H5N2	–	–	–	–
AIV	H7N3	–	–	–	–
Mycoplasma	*Mycoplasma gallisepticum*	–	–	–	–

*Abbreviations: IBV, infectious bronchitis virus; NDV, Newcastle disease virus; ILTV, infectious laryngotracheitis virus; AIV, avian influenza virus

†AviServe ILT strain

Table 3-3. Limit of detection of the singleplexed and multiplexed microsphere-based assay.

Probe		Target	DNA amount	Corresponding copy number*
Ark-P	Singleplexed	Ark-DPI	6.4ng	1.10×10^{10}
	Multiplexed	Ark-DPI	9.05ng	1.56×10^{10}
Mass-P	Singleplexed	Mass41	3.75ng	6.46×10^{9}
	Multiplexed	Mass41	4.2ng	7.24×10^{9}
Conn-P	Singleplexed	Conn	7.5ng	1.29×10^{10}
	Multiplexed	Conn	10.5ng	1.81×10^{10}
Del/GA98-P	Singleplexed	DE072	15.9ng	2.74×10^{10}
	Multiplexed	DE072	15.75ng	2.71×10^{10}
Del/GA98-P	Singleplexed	GA98	7.2ng	1.24×10^{10}
	Multiplexed	GA98	7.2ng	1.24×10^{10}

* Corresponding copy number was calculated using a following equation. (1bp= 650 Dalton)

Number of copies = [DNA amount (ng) × (6.022×10^{23})] / [(length of template (bp)) × (1×10^{9}) × 650]

Table 3-4. Comparison of the multiplexed microsphere-based assay with nucleotide sequencing analysis of clinical samples*.

Type specific probe	Microsphere-based assay results	Sequencing results		Sensitivity (%)	Specificity (%)	Agreement (%)	Kappa value
		Positive	Negative				
Ark-p	Positive	23	4†	100	89.1	93.2	0.86
	Negative	0	32				
Mass-P	Positive	9	4†	100	92	93.2	0.77
	Negative	0	46				
Conn-P	Positive	5	0	100	100	100	1.00
	Negative	0	54				
Del/GA9	Positive	10	2	100	95.9	96.6	0.88
8-P	Negative	0	47				

* Clincial samples were previously confirmed by nucleotide sequencing.

† Samples with double positive signals from the microsphere-based assay including GA08 were considered.

Table 3-5. Clinical samples with double signals of microsphere-based assay.

Sample #	Sequencing results	Microsphere-based assay results				RFLP results
		Ark-P	Mass-P	Conn-P	Del/GA98-P	
67297	GA08	Weak positive*	Weak positive	−†	−	GA08
67634	GA08	Weak positive	Weak positive	−	−	GA08
82323	Ark	Positive	Positive	−	−	Ark-DPI & Mass41
82427	Mass	−	Positive	−	Positive	Mass41 & DE072/GA98
83147	Ark	Positive	−	−	Weak positive	Ark-DPI
83188	GA08	Weak positive	Weak positive	−	−	GA08
87828	Conn	Weak positive	−	Positive	−	Conn

* Positive MFI value was considered as weak positive when it was less than three folds of cut-off value.

† − indicate negative signal.

Figure 3-1. Target serotype specificity of the probes used in this study in a singleplexed (A) and a multiplexed (B) microsphere-based assay. The median fluorescence intensity (MFI) was measured in triplicate and the error bars indicate means ± standard deviations.

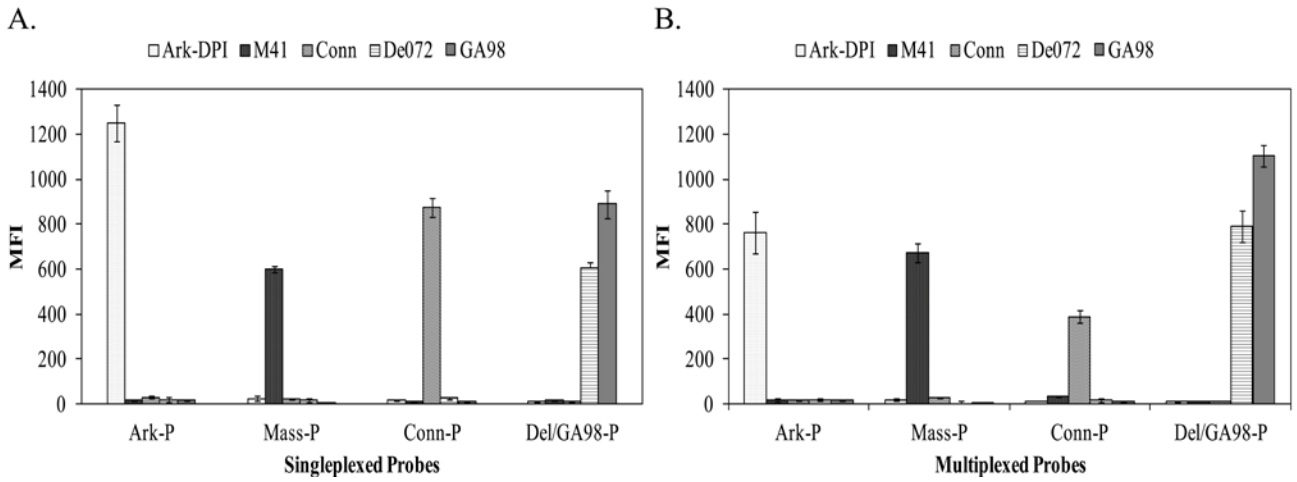
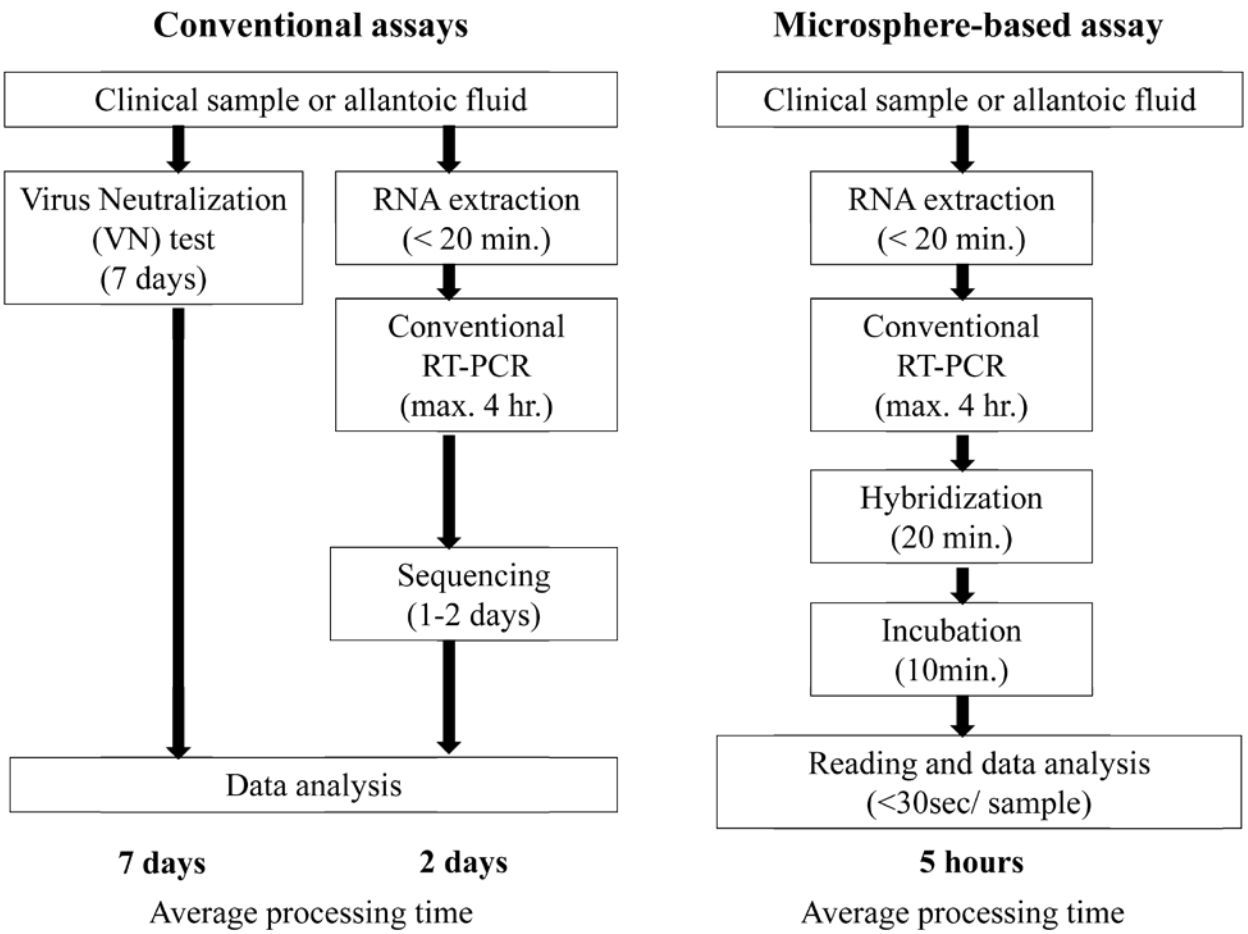

A.
Ark-DPI M41 Conn De072 GA98
MFI
1400
1200
1000
800
600
400
200
0
Ark-P
Mass-P
Conn-P
Del/GA98-P
Singleplexed Probes
B.
Ark-DPI M41 Conn De072 GA98
MFI
1400
1200
1000
800
600
400
200
0
Ark-P
Mass-P
Conn-P
Del/GA98-P
Multiplexed Probes

Figure 3-2. Comparison of the procedures and timelines of conventional assays and the microsphere-based assay for infectious bronchitis virus serotype identification.


Conventional assays
Clinical sample or allantoic fluid
Virus Neutralization (VN) test (7 days)
RNA extraction (< 20 min.)
Conventional RT-PCR (max. 4 hr.)
Sequencing (1-2 days)
Data analysis
7 days
2 days
Average processing time
Microsphere-based assay
Clinical sample or allantoic fluid
RNA extraction (< 20 min.)
Conventional RT-PCR (max. 4 hr.)
Hybridization (20 min.)
Incubation (10min.)
Reading and data analysis (<30sec/ sample)
5 hours
Average processing time

CHAPTER 4

DETECTION AND DIFFERENTIATION OF INFECTIOUS BRONCHITIS VIRUSES USING RRT-PCR[2]

[2]Roh,H-J. D.A.Hilt, and M.W.Jackwood. To be submitted to *Avian Diseases*.

Abstract

Detection and differentiation of infectious bronchitis virus (IBV) strains using real-time RT-PCR (RRT-PCR) was attempted. Using strain-specific primers and probes targeting the S1 hypervariable region, duplex RRT-PCR was performed. Specificity and limit of detection of each probe was determined using reference IBV viruses. Evaluation of clinical samples using RRT-PCR revealed that this assay has a potential to identify co-infection of IBV strains. However optimization and modification may further be required to improve the specificity and sensitivity of the assay.

Key words: infectious bronchitis virus, RRT-PCR, diagnosis, strain typing, differentiation

Abbreviations: IBV = infectious bronchitis virus, RRT-PCR = real-time reverse transcriptase-polymerase chain reaction, S1 = spike 1

Introduction

Infectious bronchitis (IB) is highly contagious upper-respiratory disease of chickens. The disease results in major economic losses in the poultry industry worldwide due to poor feed conversion, decreased egg production, and predisposition to secondary infections (2). Infectious bronchitis virus (IBV) is the causative agent of IB, which is a coronavirus in the genus *Gammacoronavirus*,. Due to high mutation rates during replication of the IBV RNA genome, more than a dozen serotypes and countless variants exist, which most often do not confer cross-protection against each other (4, 6, 13, 15). Thus, control of IBV depends on selection of vaccines based on currently circulation IBV types as well as proper vaccine administration. Because identification of relevant field serotypes is a necessity for effective vaccination protocols, rapid and accurate diagnostic methods are critically important.

Conventional IBV serotyping methods include virus isolation and virus neutralization tests using embryonated specific pathogen free (SPF) chicken eggs or organ culture. Those tests are accurate but time-consuming, laborious and often expensive, and sometimes more than a single passage in embryonated eggs or organ culture is required to propagate field viruses (5). More recently, however, molecular approaches have been extensively used for IBV identification and typing. Reverse transcriptase polymerase chain reaction (RT- PCR) targeting the S1 gene region followed by restriction fragment length polymorphism (RFLP) (10) or nucleotide sequencing has been widely used to identify and further classify IBV viruses (7-9, 11, 12, 16). In addition, real-time RT-PCR (RRT-PCR), which is more sensitive and specific than conventional RT-PCR assays, has been developed and used to detect IBV. Previous IBV RRT-PCRs were designed to target the 5’ untranslated region (UTR) of S1 gene (1) or N gene (14), with both assays able to detect IBV but not differentiate IBV strains.

Rapid and accurate detection of circulating IBV types is critical for selection of the appropriate vaccines for control of IB in commercial poultry. The RRT-PCR test is a highly sensitive and specific diagnostic assay that can also analyze clinical samples significantly faster than conventional diagnostic methods. Previously, RRT-PCR assays for IBV targeting the 5' UTR region of the S gene, which is conserved among IBVs, or the N gene which is highly conserved have been reported (1, 3, 14). A Taqman probe based RRT-PCR assay, developed by Callison *et al*., (1), was designed to detect the 5'-UTR region of the IBV genome of strains in the U.S. and differentiate IBV from other avian respiratory pathogens. However it also detected turkey coronavirus (TCoV) since the 5' UTR of TCoV shares high similarity with IBV. Chousalkar *et al*., (3) also designed RRT-PCR targeting the 5'-UTR region of the genome, using a short fluorescent dye labeled locked nucleic acid probe to detect viral load of Australian IBV strains, A3 and VicS, from the oviduct of hens. Another Taqman probe based RRT-PCR assay was designed by Meir *et al*., (14). That assay targeted the N gene of IBV, which is highly conserved among IBV strains and also abundant in infected cells. This RRT-PCR assay was sensitive enough to detect the virus directly from tracheal swabs and was specific to IBV by not detecting any other avian respiratory pathogens including TCoV.

In this study, our objective was to rapidly differentiate commonly used IBV vaccine strains. We designed RRT-PCR assays targeting the S1 gene of Arkansas, Massachusetts, Connecticut, Delaware and GA98 and describe the sensitivity and specificity of the RRT-PCR assay for detection and differentiation of IBV.

Materials and Methods

Primers and Probes

Primers and probes (Table 4-1) were designed against the hypervariable region in the S1 gene. Targeted serotypes in this study were Arkansas, Massachusetts, Connecticut, DE072 and GA98, which are commonly used IBV vaccine types and the most frequently isolated serotypes in the U.S. Currently available IBV sequences in GenBank were aligned using the ClustalW method in DNASTAR and regions specific to each serotype were identified to design serotype specific probes. Forward and reverse primers were designed for each serotype to generate an amplicon less than 200bp in length. We performed a BLAST analysis (www.ncbi.nlm.nih.gov) to verify the specificity of probes and primers. Minor groove binding (MGB) probes were obtained from Applied Biosystems (Foster City, CA, USA) and black hole quencher (BHQ) probes and primers were obtained from Integrated NDA Technologies (Coralville, IA).

Virus

Previously identified known IBV viruses were used for initial optimization of the assay. Arkansas DPI (Ark-DPI), Massachusetts 41 (M41), Connecticut (Conn), Delaware 072 (DE 072), and Georgia 98 (GA98) were propagated in 9-10 day-old specific pathogen-free (SPF) embryonated chicken eggs and the 50% embryo infectious dose titer (EID_{50}) was calculated by Reed and Muench method. Titers were 1×10^{7} EID_{50}/ml for Ark-DPI, $1 \times 10^{7.3}$ EID_{50}/ml for M41, 1×10^{7} EID_{50}/ml for Conn, $1 \times 10^{5.5}$ EID_{50}/ml for DE072 and 1×10^{6} EID_{50}/ml for GA98. Other chicken respiratory pathogens including infectious laryngotracheitis virus (ILTV), Newcastle disease virus (NDV) Lasota strain, avian influenza virus (AIV) H5N2 and H7N3, and *Mycoplasma gallisepticum* (MG) used in this study were obtained from the Poultry Diagnostic

and Research Center (PDRC), Athens, GA. For evaluation of the assay, 59 clinical samples collected from chickens were obtained from Dr. Holly S. Sellers at PDRC.

RNA Extraction

Viral RNA was extracted from 50 ul of allantoic fluid or clinical samples using the MagMax96 total RNA isolation kit (Ambion, Austin, TX) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturer's protocols.

RRT-PCR condition

The AgPath-ID™ One-step RT-PCR kit (Ambion Inc., Austin, TX) were used to perform RRT-PCR assays according to the manufacturer's instructions. The 25µl RRT-PCR reaction mixture included 12.5µl 2X RT-PCR buffer, 10µM of each primer, 4 µM of each probe, 1µl of 25X RT-PCR enzyme mix, and 2.5µl of viral RNA. The RRT-PCR reaction was performed on the SmartCycler II (Cepheid, Sunnyvale, CA) under the following conditions: one cycle of 50°C for 30min and 95°C for 15min followed by 40 cycles of 94°C for 1sec and 60°C for 60sec (for Ark-P and Mass-P duplex RRT-PCR), or 40 cycles of 94°C for 1sec and 53°C for 60sec (for Conn-P and Del/GA98-P duplex RRT-PCR).

Specificity and Limit of detection

Specificity of the RRT-PCR assay was tested against RNA extracted from known avian respiratory pathogens including other serotype infectious bronchitis viruses (GA08, Iowa, Florida, JMK, Gray, Holte, Califonia 99), AIV (H5N2 and H7N3), ILTV, NDV and MG. We prepared 10-fold serial dilutions of allantoic fluid from known control viruses to estimate the amplification efficiency and the limit of detection of the RRT-PCR assays.

Results and Discussion

The RRT-PCR assays reported to date are only able to distinguish IBV from other chicken respiratory pathogens, they cannot differentiate IBV strains. The objective of this work was to design a RRT-PCR assay to differentiate IBV serotypes which are highly prevalent in the U.S. namely; Ark, Mass, Conn, DE072 and GA98. Primers and probes for each serotype targeting the S1 hypervariable region were designed (Table 4-1), and the RRT-PCR assays were performed as a duplex with Ark-P and Mass-P together, or Conn-P and Del/GA98-P together in a single tube. Specificity of each probe was verified using known IBV viruses. In addition to targeted serotypes, Iowa, Florida, JMK, Gray, Holte, California 99 and GA08 type IB viruses were tested and each probe only specifically detected targeted IBV serotypes. Also, no other avian respiratory pathogens, including NDV, ILTV, AI (H5N2 and H7N3) and MG, were detected in the assay. The specificity of this RRT-PCR was found to be high for the target serotype of IBV. The limit of detection of each probe was determined using 10 fold serial dilutions of allantoic fluid of known reference virus and the last dilution of virus detected by each probe was listed in Table 4-2. Mass-P and Ark-P was more sensitive than other two probes used in RRT-PCR.

Clinical samples taken from chickens with clinical signs of IBV were previously typed by RT-PCR and nucleotide sequencing. Results from clinical sample evaluation using RRT-PCR are shown in Table 4-3. All four probes showed more than 95% specificity. Among all probes, sensitivity of Conn-P was lowest at 80%, although this could partly be due to the small sample size for Conn positive samples (n=5).

Sample 82427 was previously identified as Mass by nucleotide sequencing, and detected by Mass-P as well as Del/GA98-P in the RRT-PCR assay. To verify this result, we used the

restriction fragment length polymorphism (RFLP) test, which revealed a co-infection of Mass and DE072 (or GA98) in sample 82427. Sample 87828, identified as Conn by nucleotide sequencing, was not detected by Conn-P. It is not clear why this sample was negative. It is possible that either the probe target region on this virus had mutations preventing hybridization or the virus titer in this sample was lower than the limit of detection of Conn-P. Some IBV positive non Ark or non Mass samples were positive with the Ark-P or Mass-P having relatively low C_T values (>36). That result could be due to cross reactions with Ark-P or Mass-P and the IB viruses in the clinical samples. However the possibility of persistence of vaccine viruses or existence of multiple serotypes in samples should also be considered since these clinical samples were obtained from vaccinated birds.

Currently multivalent IBV vaccines are routinely used in the field to provide a wide range of protection against different IBV serotypes and more than one IBV type can be found in clinical samples. A test that can quickly and quantitatively distinguish more than one virus type in clinical sample is needed to control the disease. By targeting serotypes most frequently used in vaccine, this test will cover more than 90% of serotypes seen in the diagnostic laboratory. Information acquired from accurate and timely detection of circulating field viruses will help with selection of vaccine combinations that provide better protection.

Advantages of the RRT-PCR test are simple processing steps and quick assay readout. A disadvantage of the RRT-PCR assay, however, was diagnostic sensitivity that was slightly lower than other conventional assay. Once improved by additional optimization, the simplicity and speed of this diagnostic test coupled with the ability to identify multiple serotypes in a single clinical sample will make this assay an important method for researchers to obtain more detailed information of IBV infection.

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Table 4-1. Primers and probes used in this study

Name of primers/probes	Target serotype	Sequences (5' →3')	References
Ark-F'	Arkansas	GGTGAAGTCACTGTTTCTA	Roh et al 2013
Ark-R'		AGCACTCTGGTAGTAATAC	
Ark-P		**TET**-TRTATGACAACGAATC-**MGBNFQ**	
Mass-F'	Massachusetts	CGTKTACTACTAYCAAAGTGC	This study
Mass-R'		CCATGAATARTACCAACARTACAC	
Mass-P		**FAM**-AGCCTGCATTATTARAT-**MGBNFQ**	
Conn-F'	Connecticut	ATGCRGTAGTTAATACTTC	This study
Conn-R'		CGWCATAGCTATAGARGAA	
Conn-P		**CY5**-ACCAATAATACCAACAATACACTCTCTTAA-**BHQ-2**	
Del/GA-F'	Delaware & Georgia 98	AGGCGTTTGTACTGYATA	This study
Del/GA-R'		GCCATGCCTTAAAATTTG	
Del/GA-P		**TET**-TTKTGACACACTGTGGT-**MGBNFQ**	

Table 4-2. RRT-PCR assay limit of detection.

Probe	**Virus**	**Initial titer**	**Limit of detection (singleplex)**
Ark-P	Ark-DPI	1×10^{7} EID_{50}/ml	10^{-6} dilution
Mass-P	M41	$1\times10^{7.3}$ EID_{50}/ml	10^{-7} dilution
Conn-P	Conn	1×10^{7} EID_{50}/ml	10^{-4} dilution
Del/GA98-P	DEL072	$1\times10^{5.5}$ EID_{50}/ml	10^{-4} dilution
	GA98	1×10^{6} EID_{50}/ml	10^{-4} dilution

Table 4-3. Evaluation of clinical samples using RRT-PCR assay.

Type specific probe	RRT-PCR result	Sequencing result*		Sensitivity (%)	Specificity (%)
		Positive	Negative		
Ark-P	Positive	20	2	86.9	93.5
	Negative	3	29		
Mass-P	Positive	9	2	100	95.5
	Negative	0	43		
Conn-P	Positive	4	0	80	100
	Negative	1	49		
Del/GA98-P	Positive	9	1	100	97.7
	Negative	0	44		

*Clinical samples were previously tested with the nucleotide sequencing.

CHAPTER 5

EVALUATION OF IBV ARK TYPE VACCINE FAILURE IN COMMERCIAL BROILERS[3]

[3]Roh, H-J. D.A.Hilt, S.M.Williams, and M.W.Jackwood. Accepted by *Avian Diseases*.

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Abstract

Infectious bronchitis virus (IBV) causes an upper-respiratory tract disease in chickens and is highly contagious. Many different types of the virus exist, but only a few types are used as attenuated live vaccines in the commercial poultry industry. Of the vaccine types used, the Arkansas (Ark) type virus is most frequently re-isolated from vaccinated broilers. Previous research has suggested that incomplete clearance of Ark type vaccine virus plays a role in the inadequate protection observed when vaccinated broilers are challenged with pathogenic Ark virus. In this study we examine routes of vaccine administration using multiple IBV types including Ark in an effort to understand why Ark vaccines do not provide good protection and persist in commercial broilers. We found that interference between different types of IBV vaccines was not occurring when combined and administered using a commercial hatchery spray cabinet. Also, Ark vaccine virus was not efficacious in 1-day-old broilers when sprayed using a hatchery spray cabinet but gave good protection when administrated by eye drop. We also found that the amount of Ark vaccine virus was low or undetectable in choanal swabs out to 35 days post-vaccination when vaccine was administered by eye drop or drinking water. Alternatively, a subpopulation of the Ark vaccine isolated from a vaccinated bird (Ark-RI-EP1) showed a peak titer at 7 to 10 days of age when given by the same routes, suggesting that the Ark-RI-EP1 was more fit with regard to infection and/or replication in the birds. Moreover, we found that detection of IBV vaccine virus early after administration (regardless of strain or route) correlated with protection against homologous challenge, which may be a good indicator of vaccine efficacy in the field since humoral antibody titers are typically low or undetectable following vaccination. These experiments provide some key findings that can be used to direct the efforts

for improving the efficacy of IBV Ark type vaccines given in the hatchery and are an important step in elucidating the factors contributing to the persistence of Ark vaccine in the field.

Key words: infectious bronchitis virus; avian coronavirus; Arkansas vaccine; failure.

Abbreviations: Ark-DPI= Arkansas-Delmarva Poultry Industry; IBV= infectious bronchitis virus; ORF= open reading frame; PDRC= Poultry Diagnostic and Research Center; RT-PCR= reverse transcriptase polymerase chain reaction

Introduction

Avian infectious bronchitis virus (IBV) is a highly infectious pathogen of chickens that primarily infects epithelial cells of the upper respiratory tract. Infection causes various clinical signs including nasal discharge, sneezing, watery eyes, weight loss and lethargy. Depending on virus cell tropism, epithelial cells in the kidney or oviduct can be infected, causing nephritis and decreased egg production, respectively (5). Mortality caused solely by IBV is generally low compared to other avian viral pathogens, but young chicks predisposed to IBV infection are more susceptible to secondary bacterial infections which can be lethal (6). IBV is distributed worldwide and costs the poultry industry millions of dollars annually through production decreases, condemnations at the processing plant and the high cost of prevention measures, including vaccination.

IBV is a gamma coronavirus, in the family *Coronaviridae*, and within the order *Nidovirales*. It is an enveloped virus with a single-stranded positive-sense RNA genome approximately 27 to 28 kb in length. The viral genome encodes the viral replicase complex (1a and 1ab encoding nonstructural proteins 2 to 16), structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N), as well as several nonstructural proteins. The S protein is a large glycoprotein projected from the virus envelope that consists of an amino terminal S1 subunit, making up the apical surface of the S protein, and a carboxyl terminal S2 subunit, which anchors the S protein to the viral membrane. The S protein is responsible for viral attachment to cell receptors and virus-host membrane fusion. The location and makeup of the receptor binding domain in S1 varies among other coronaviruses, but has not been identified for IBV (5).

Since the first identification of IBV in the 1930s in the USA (reviewed in (11)), various serotypes and strains with antigenic variation have been found worldwide (5). There is little to no

cross protection between serotypes or circulating variant viruses, making it extremely difficult to control the disease (10). Different virus types are the result of mutations and recombination in the spike protein during virus replication. Mutation and/or recombination in spike, which can change the epitopes that induce neutralizing antibodies, allows new virus types to infect and cause disease even in vaccinated birds.

Currently, the best strategy for control of infectious bronchitis (IB) is the use of live-attenuated vaccines, despite the fact that vaccinated birds do not obtain cross-protective immunity against heterologous viruses. In broilers in the USA, live vaccines are typically given at one day of age in the hatchery using a spray cabinet and at approximately two weeks of age in the field by an aerosol sprayer or in the drinking water. In addition, multiple serotypes are combined together and used for vaccination in an attempt to induce broader protection (7). Live vaccines stimulate both humoral and cellular immune responses (5), which can cause a vaccine reaction if not administered properly. Vaccine strains of IBV have also been reported to revert to pathogenicity (14). Therefore, diagnosis of circulating viruses and choosing and properly administering the right vaccine type are critical for the control of IBV.

It is generally accepted that birds vaccinated in the hatchery develop sufficient immunity to clear the field boost vaccine virus from the upper respiratory track by 5 days post vaccination (19). However, this does not apply to all IBV serotypes as persistence of the Arkansas (Ark) type vaccine, one of the most widely used vaccine serotypes in the USA, was also reported (19). The persistence of Ark type vaccines in commercial broilers can provide the virus with opportunities to undergo mutations, which can result in a pathogenic phenotype capable of causing a disease outbreak. To this end, variant Ark viruses (Ark-like viruses) have been reported (1, 16, 17, 23, 24) in vaccinated birds, indicating that the virus is changing. It is not clear why Ark type

vaccines are persisting in commercial broilers; however, it may be due to inadequate priming of the immune response by hatchery vaccination. In this study we examine different routes of vaccine administration as well as vaccination with multiple IBV types including Ark, in an effort to understand why Ark vaccine viruses persist in commercial broilers.

Materials and Methods

Vaccines and challenge viruses

Commercially available mono and bivalent live attenuated vaccines of the Ark, Mass, and GA98 types were used in this study. Dr. J. Gelb, Jr. (University of Delaware, Newark, DE) kindly provided the pathogenic Arkansas-Delmarva Poultry Industry (Ark-DPI) Ark/Ark-DPI/81 and the Massachusetts (Mass 41) Mass/Mass41/41 strains. The pathogenic Georgia 98 (GA98) virus, GA98/CWL0470/98, was isolated in our laboratory in 1998 (20).

Chickens

Commercial non-vaccinated broiler chicks were obtained from a commercial source at 1 day of age and maintained in positive-pressure Horsfal isolation units at the Poultry Diagnostic and Research Center (University of Georgia). Feed and water were provided *ad libitum*.

Experiment 1

To examine if interference occurs between Ark vaccine viruses and other IBV vaccine serotypes, we immunized birds with Mass or GA98 vaccines in combination with the Ark vaccine at the manufacturer's recommended dose. Briefly, vaccine stock from the manufacturer was rehydrated in PBS (1000 doses/ml). Working solutions were then prepared so that the proper number of vaccine doses (1dose/bird) were mixed with PBS in a total volume of 7ml. Working vaccine solution titers were 1 X $10^{4.6}$ 50% egg infectious doses (EID_{50})/ml for Ark vaccine, 1 X

$10^{4.5}$ EID_{50}/ml for Mass vaccine and 1 X 10^5 EID_{50}/ml for GA98 vaccine. 1-day-old broilers were divided into 6 groups (Ark vaccine group, Mass vaccine group, GA98 vaccine group, Ark and Mass combined vaccine group, Ark and GA98 combined vaccine group, and a non-vaccinated control group), and vaccinated using a commercial hatchery spray cabinet delivering 7ml of vaccine suspension in a single application. Tracheal swabs and tears were collected from 5 birds in each group at 3, 7, 10, 14, 17, 21, and 28 days of age. Samples were collected from different birds at each time point. At 30 days of age, 5 birds from each group were challenged intraocularly and intranasally with pathogenic Ark-DPI (1 X 10^5 EID_{50}/bird), Mass 41 (1 X $10^{5.3}$$EID_{50}$/bird), or GA98 (1 X $10^5$$EID_{50}$/bird), and 5 birds were maintained as non-challenge controls. At 5 days post-challenge, all birds were examined for clinical signs, tracheal swabs and tears were collected for virus detection, and sera was collected and examined for antibodies against IBV using a commercial ELISA kit (IDEXX, Portland ME). Euthanized birds were examined for lesions and tracheal tissues were collected for histopathology. Real-time RT-PCR was used to determine the presence of vaccine and challenge virus in tracheal swabs and tears. Tracheal tissue samples were fixed in 10% neutral buffered formalin, routinely imbedded in paraffin, sectioned, and stained for histopathologic examination. Tracheal tissues were prepared and the lesions were scored from 1 to 4 (1 = normal, 2 = focal, 3 = multifocal, and 4 = diffuse) as previously described (15).

Experiment 2

To determine if dose affects the efficacy of Ark vaccine when delivered using a hatchery spray cabinet, we tested 2 different doses of Ark vaccine with different numbers of 1-day-old broilers. To ensure that the vaccines were evenly sprayed on all birds, we used only one nozzle in the spray cabinet, and made a circular shaped cardboard enclosure approximately 34 cm in

diameter that confined a maximum of 40 one-day-old chicks to the spray range of the nozzle. The birds were divided into 5 groups; 15 birds in the no vaccine control, 20 birds given 20 doses of vaccine, 20 birds given 40 doses of vaccine, 40 birds given 40 doses of vaccine, and 40 birds given 80 doses of vaccine. Birds in each of the vaccinated groups were sprayed with 7ml of Ark vaccine suspension. Each group of chicks was housed in separate isolators for 1 hour after vaccination and 15 birds per group were maintained. The remaining chicks were sacrificed. Choanal swabs and tears were collected at days 3, 7, 10, 14, 17, 21, and 28 days of age from the same birds in each group. At 30 days of age, 10 birds from each group were challenged via the intraocular / intranasal route with pathogenic Ark-DPI (1 X 10^5 EID_{50}/bird) and 5 birds per group were kept as non-challenge controls. At 5 days post-challenge, choanal swabs and tears were collected from all of the birds for virus detection by real-time RT-PCR analysis.

Experiment 3

This experiment was designed to determine if Ark vaccine re-isolated from vaccinated broilers at 21 days post-vaccination has an advantage over the original commercial vaccine with regard to infection and replication in broiler chicks. The Ark vaccine re-isolated at 21 days post-vaccination was passed in embryonated eggs one time to increase the virus titer and the resulting virus isolate was designated Ark-reisolated-egg pass 1 (Ark-RI-EP1). The commercial Ark vaccine and Ark-RI-EP1 virus were titered in embryonated eggs. Replication of the viruses was examined by giving the Ark vaccine or Ark-RI-EP1 (1 X $10^{4.5}$ EID_{50}/bird) to 10 one day old broilers by eye drop. A non-vaccinated negative control group was also maintained. Choanal swabs and tears were collected from the same birds (10 birds per each group) at 1, 3, 7, 10, 14, 17, 21, 28, and 35 days of age and analyzed for virus replication using real-time RT-PCR. The nonstructural protein 3 (nsp 3) gene and S1 gene sequences of the Ark vaccine and Ark-RI-EP1

were determined and consensus sequences were compared with the previously published Ark vaccine viruses to identify changes.

Experiment 4

To determine if vaccine application methods affect vaccine efficacy, we inoculated 1 day old broilers with either the Ark vaccine or Ark-RI-EP1 by spray, drinking water or eye drop. Drinking water vaccine was prepared in cold distilled water with 0.1% powdered skim milk as a stabilizer and was consumed by the birds within 1 hour. The Ark vaccine and Ark-RI-EP1 virus were titered and a dose of 1 X $10^{3.4}$ EID_{50}/bird was given by spray, eye drop or via drinking water. Choanal swabs were collected from the same birds (10 birds per each group) at 3, 7, 10, 14, 17, 21, and 28 days of age. At 30 days of age, 10 birds in each group were challenged via the intraocular/ intranasal route with pathogenic Ark-DPI (1 X $10^5$$EID_{50}$/bird), and 5 birds were kept as negative challenge controls. At 5 days post-challenge, choanal swabs and tears were collected for virus detection by real-time RT-PCR, serum was collected and tested for antibodies to IBV by ELISA (IDEXX), and tracheas were collected and fixed in 10% buffered formalin for histopathologic analysis as described above.

Virus detection using real-time RT-PCR

Viral RNA was extracted from swabs and tears using the MagMax96 total RNA isolation kit (Ambion, Austin, TX) and the KingFisher Automated Nucleic Acid Purification machine (Thermo Electron Corporation, Waltham, MA) according to the manufacturer's protocols. Real-time RT-PCR analysis was performed using the SmartCycler II (Cepheid, Sunnyvale, CA) and the AgPath-ID™ One-step RT-PCR kit (Ambion Inc., Austin, TX) according to the manufacturer's recommendations. The IBV specific primers and probe for the real-time RT-PCR were previously published by Callison et al. (4); forward primer IBV5'GU391 (5'-GCT TTT

GAG CCT AGC GTT-3'), reverse primer IBV5'GL533 (5'-GCC ATG TTG TCA CTG TCT ATT G-3'), and Taqman® dual-labeled probe IBV5'G (5'-FAM-CAC CAC CAG AAC CTG TCA CCT C-BHQ-3'). Ark specific probe and primers were also designed and used in this study; Ark-F' (5'-GTG AAG TCA CTG TTT CTA-3'), Ark-R' (5'-AGC ACT CTG GTA GTA ATA C-3'), and a labeled minor groove binding (MGB) probe Ark-P (5'-TET-TRT ATG ACA ACG AAT C-MGBNFQ-3'). The specificity of the Ark primers and probe were verified against Mass, GA98, Conn and DE072 IBV types (data not shown) and the assay standard curve for Ark specific probe and primers was generated by plotting the C_T values and log10 of virus copy numbers ($y=-0.2709\ x + 11.9463$; y= log10 of virus copy number, x = C_T value) with $R^2 = 0.98$. The primers were obtained from Integrated DNA Technologies (Coralville, IA), and Taqman® probe was synthesized by BioSearch Technologies (Novato, CA). The MGB probe was obtained from Applied Biosystems (Foster city, CA). Real-time RT-PCR components and thermocycler parameters were conducted as previously described, and a standard curve for the assay, which was previously published, was used to calculate the approximate genome copy number for each sample (4).

Sequence analysis of the S1 and the nsp3 genes

The Ark vaccine and Ark-RI-EP1 S1 genes were amplified by RT-PCR using previously published primers; NEWS1OLIGO5'(18), and Degenerate3'(19). For amplification of the nsp 3 gene, 2 sets of primers were designed and designated; NSP3-1-F' (5'-ACT ATA TGT TCT TCC GCT TCA -3'), NSP3-1-R' (5'- CTT CAC AAT TCT TAA CCC CAC AGT -3'), NSP3-2-F' (5'-GAT GCT AAT TGG CTT CTT G -3'), and NSP3-2-R' (5'- AGG GTT TTC TTT CTG TTT GTG TC -3'). Sequencing reactions for S1 genes were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystem, Foster City, CA) and purified using the

Performa DTR Ultra Dye Terminator removal system (Edge Biosystems, Gaithersburg, MD) according to manufacturer's protocol. Nucleotide sequencing was conducted by the Georgia Genomics Facility (University of Georgia, Athens, GA). For nsp 3 genes, gel-purified RT-PCR products were sent to GENEWIZ® (GENEWIZ inc., South Plainfield, NJ) for nucleotide sequencing. The S1 and the nsp 3 sequences for each virus were assembled using SeqMan and MegAlign programs (DNASTAR, Inc., Madison, WI).

Statistical analysis

The data were analyzed using JMP statistical Discovery Software (SAS Institute, Inc., v.9, Cary, NC). Genome copy number and C_T values are presented as mean ± standard error mean (S.E.M). Means were compared by student's t-test (α=0.05). Histopathology scores were analyzed by Kruskal-Wallis test for multiple comparisons test followed by a Dunns post test. Significance is reported at the level of $p < 0.05$.

Results

Experiment 1

Birds in this experiment were spray vaccinated at 1 day of age, challenged at 30 days of age and the average viral genome copy number per group, analyzed by real-time RT-PCR using IBV-5'G probe, was calculated based on the previously published standard curve for this real-time RT-PCR assay (4), and the data are shown in Fig. 5-1. Tracheal swabs and tears collected from vaccinated and non-challenged birds at 35 days of age (5 days post challenge) from each vaccine group were also analyzed to evaluate vaccine virus replication. Replication of Ark vaccine was not detected until 21 days of age, after which it quickly declined in both tracheal swabs and tears (Fig.5-1A and C). The peak titer of Mass, and GA98 vaccines given alone (Fig.

5-1A and C) or in combination with Ark vaccine (Fig.5-1B and D) occurred between 10 to 14 days of age. To detect the Ark vaccine virus in combination with other vaccines, an Ark specific probe (Ark-P) was used and the average viral genome copy number was calculated based on the linear standard curve. The relative amount of Ark vaccine virus is presented in Fig.5-2. In contrast to birds given Ark vaccine alone, which showed a peak titer between 21 and 28 days of age, birds given a combined vaccine of either Ark with Mass or Ark with GA98, had small amounts of the Ark vaccine in both tracheal swabs and tears, though often times below the limit of detection for this assay. Only in tracheal swabs from birds given Ark and GA98 vaccines combined the Ark vaccine was detected at 7 days of age, but no virus was detected at any other time point.

Clearance of the challenge virus in vaccinated and non-vaccinated birds was determined by real-time RT-PCR on RNA extracted from tracheal swabs and tears collected at 5 days post-challenge (Fig.5-3). Birds vaccinated with Ark and challenged with the homologous virus (Fig.5-3A and C) were not adequately protected, as indicated by detection of high amounts of viral RNA, and no statistically significant difference was observed between that group and non-vaccinated birds challenged with the same virus. Birds vaccinated with either Mass or GA98 were protected against homologous challenge (Fig.5-3A and C). In combined vaccine groups, birds were adequately protected against the homologous Mass or GA98 virus, and also showed a slightly better protection against Ark-DPI challenge compared to that of birds vaccinated with Ark alone (Fig.5-3B and D). In most groups, virus load detected in tears was 10 to 100 fold higher than tracheal swabs. The number of virus positive birds, corresponding C_T values and histopathology scores for each group are shown in Table 5-1. Vaccine virus was detected in most of the vaccinated groups on the two days before challenge (28 days of age), though at a very low

level close to the limit of detection, and birds that were vaccinated and not challenged were also positive for virus at necropsy (35 days of age) indicating that vaccine virus was still present. The C_T values in the non-vaccinated/challenged groups were between 22.8 and 26.4, whereas the vaccinated and challenged groups had higher C_T values indicating fewer viruses were present. The histopathology scores of groups challenged with Ark-DPI were higher than any other group and showed no statistical difference with birds in the non-vaccinated-challenge groups.

All of the sera collected from birds at 1 day of age were positive for maternal antibodies. To determine if the maternal antibodies were neutralizing, Ark, Mass, and GA98 vaccines were used in virus neutralization (VN) tests in embryonated eggs. Good protection against both Ark (average titer = 91.2) and Mass (average titer= 97.7) was observed and comparatively low maternal antibodies against GA98 were observed (average titer = 4.79). There was little or no specific IBV humoral antibody detected at 5 days post-challenge, in any of the groups (data not shown).

Experiment 2

In this experiment, 5 groups of either 20 or 40 birds were given a 1X or 2X dose of vaccine by hatchery spray cabinet and vaccine virus levels in tracheal swabs and tears were measured by real-time RT-PCR from 3 through 28 days of age. A low level of vaccine virus was detected at 21 days of age in 2 and 6 of 15 birds in choanal swabs and tears respectively in the group of 20 birds that received 40 doses of vaccine. Vaccine virus was not detected in any other group prior to challenge.

The birds were challenged at 30 days of age and the clinical signs and clearance of the homologous Ark-DPI challenge virus are presented in Table 5-2. Clinical signs at 5 days post-challenge were observed in$\geq$ 80% of birds in all of the groups except the group where 40 doses

of Ark-DPI vaccine were given to 20 birds, which had only 2/10 birds positive for clinical signs. The Ark-DPI challenge virus was detected in all of the birds in all groups except the non-challenged negative control group; however, in the group of 20 birds that received 40 doses of vaccine, higher C_T values in both choanal swabs and tears were seen, indicating less challenge viruses was present compared to the other challenge groups.

Experiment 3

In this experiment, the levels of commercial Ark vaccine and the vaccine virus re-isolated from broilers at 21 days post-vaccination and passed 1 time in embryonated eggs (Ark-RI-EP1) were examined in eye drop vaccinated birds by real-time RT-PCR, and the data are shown in Fig. 5-4. An equal dose of both vaccines was given. The levels of Ark vaccine peaked at 3 days of age followed by a gradual decline until 35 days of age. The highest Ark-RI-EP1 virus levels were at 1 day of age then declined until 10 days of age. A slight rise in copy number was observed for the Ark-RI-EPI virus at 14 days of age. Both viruses were below the limit of detection by 35 days of age when the experiment was terminated. This data is in contrast to the Ark vaccine replication pattern in experiment 1 (Fig.5-1.), where Ark vaccine was administrated using a hatchery spray cabinet and not detected until 21 days of age.

Experiment 4

In this experiment, different groups of birds vaccinated by a hatchery spray cabinet, drinking water or eye drop using Ark vaccine or Ark-RI-EP1 and were monitored for the level of vaccine virus by real-time RT-PCR. The data are shown in Fig.5-5. Low levels of the Ark vaccine virus (1×10^2 to $1 \times 10^{2.5}$ genome copies) were detected in choanal swabs and tears from birds vaccinated by eye-drop (Fig. 5-5A and C), with a slight rise observed at 17 and 21 days of age. The Ark vaccine virus was not detected in the drinking water and spray-vaccinated

groups (Fig. 5-5A and C). In birds given the Ark-RI-EP1 virus via drinking water or eye-drop, the replication pattern peaked between 7 to 10 days of age then declined to undetectable levels by 21 days of age (Fig. 5-5B and D). However, no Ark-RI-EP1 virus was detected from either choanal swabs or tears in the spray vaccinated birds (Fig.5-5B and D).

To assess protection, we challenged the birds at 30 days of age with pathogenic Ark-DPI (Table 5-3 and Fig. 5-6). None of the vaccinated and non-challenged birds had clinical signs or significant histopathology lesions. A small amount of virus was detected in one non-challenged bird vaccinated via the drinking water. In the birds vaccinated with Ark by eye drop and challenged with Ark-DPI, only 1 out of 10 birds showed clinical signs, whereas the spray and drinking water vaccinated and challenged groups had 8 of 10 and 9 of 10 birds with clinical signs respectively. A low level of challenge virus was observed in choanal swabs and tears in the group given Ark vaccine virus by eye drop (Fig. 5-6A and C), whereas relatively high levels of virus, which were statistically different from the eye drop vaccinated group, were found in the spray and drinking water vaccinated and challenged groups. Histopathology scores of all the challenged birds vaccinated with Ark were significantly different from the negative control group, with the exception of the group vaccinated by eye drop.

Groups given the Ark-RI-EP1 virus by eye drop or drinking water and challenged with Ark-DPI had fewer birds with clinical signs (0 and 2 of 10 respectively) when compared to the challenged birds vaccinated with Ark-RI-EP1 by spray (8 of 10 with signs). Low levels of virus were detected in 2 non-challenged birds from each of the eye drop and drinking water vaccinated groups. Challenge virus was detected in all of the challenged groups but birds that received the Ark-RI-EP1 virus by eye drop or in the drinking water had fewer positive birds (4 and 6 of 10 respectively) and significantly less virus was detected in those groups compared to the other

challenge groups (Fig. 5-6 B and D). Histopathology scores were statistically significant only in the birds receiving no vaccine and challenged or vaccinated by spray and challenged when compared with negative controls. Based on histopathology, all other groups were protected.

Sequence analysis

The S1 and the nsp 3 gene sequences for the commercial Ark vaccine and the Ark-RI-EP1 virus are shown in Table 5-4. For S1, previously published subpopulations were compared and the most closely related sequence was included with our viruses in Table 5-4. The Ark-RI-EP1 S1 gene sequence had 9 non-synonymous point mutations and a 3 nucleotide deletion compared to Ark vaccine. For the nsp 3 gene, there was only a single non-synonymous mutation between Ark-RI-EP1 and the Ark vaccine.

Discussion

In this study, we examined the lack of protection observed in birds given a commercially available Ark vaccine by hatchery spray cabinet. We conducted 4 experiments with the first experiment designed to examine if other IBV vaccine types are interfering with the Ark vaccine when given simultaneously. To distinguish Ark vaccine virus when it was combined with Mass or GA98 vaccine, two separate probes, a universal IBV probe (IBV-5'G) and an Ark type specific probe (Ark-P), were used. The second experiment was designed to determine if a more focused spray at either a 1X or 2X dose would improve the efficacy of the Ark vaccine given by hatchery spray cabinet. There is also a possibility that a subpopulation of the vaccine, as reported by van Santen and Toro (29), would be more fit for replication in the birds and perhaps produce better immunity. Thus, the third and fourth experiments were designed to examine an isolate of

Ark vaccine obtained from broilers at 21 days post-vaccination for infection, replication and efficacy against Ark challenge in chicks.

In experiment 1, we found no interference between Ark vaccine and other vaccine types (Mass or GA98) when bivalent vaccines (Ark & Mass or Ark & GA98) were used, based on protection level. In fact, birds vaccinated with bivalent vaccines acquired better protection against pathogenic Ark-DPI virus than birds vaccinated with Ark alone. In addition, Ark vaccine virus replication slightly peaked at 7 days of age in tracheal swabs when combined with GA98, whereas the peak of virus replication was at 21 to 28 days of age when Ark vaccine was given alone. In bivalent vaccine groups, the predominant vaccine virus detected was either Mass or GA98. The earlier peak titers for the Ark vaccine when that vaccine was given in combination with GA98 could explain the slightly better protection against Ark-DPI challenge observed in those birds. It is not clear why the Ark vaccine replication peak occurred earlier when given in combination with GA98, but it is possible that a synergistic effect occurred where the GA98 vaccine created a suitable environment for infection and/or replication of the Ark vaccine. In addition, the better protection against pathogenic Ark-DPI viruses in bivalent vaccine groups supports a possible synergistic effect. Birds with a vaccine virus replication peak between 7 and 10 days of age showed better protection against homologous challenge regardless of vaccine type. It may be possible to apply this observation to assess protection against IBV in the field, since little or no ELISA titers are observed following vaccination.

In our experiments, low levels of vaccine viruses were still present in the tracheas and tears of some birds by 35 days of age regardless of vaccine type or delivery method. Alvarado *et al*. (1) showed Ark vaccine virus was detected in the trachea and the cecal tonsils up to 28 days post-vaccination in hatchery spray vaccinated broilers. Naqi *et al*. (23) also reported the shedding

of IBV Mass type vaccine virus up to 63 and 77 days after the initial exposure via the ocular route. In addition, Jackwood *et al*. (17) reported IBV vaccine was not fully cleared in commercial broilers with field boost vaccination, and only Ark vaccine was consistently identified in the vaccinated birds. These studies reinforce the importance of proper vaccination, however, even under the best condition, hatchery spray cabinet delivery of the Ark vaccine may still result in persistence of the vaccine viruses in the birds.

As expected, maternal antibodies were detected in the commercial broiler chicks at 1-day of age. Although reports in the literature indicate that maternal antibodies to IBV do not interfere with IBV vaccination at 1 day of age (8, 9), it is possible that high neutralizing maternal antibodies specific for the Ark virus could affect efficacy of that vaccine. Nonetheless, we conducted virus neutralizing (VN) tests and confirmed that neutralizing maternal antibodies were indeed present for the Ark, Mass and GA98 viruses. Since neutralizing maternal antibodies were detected for all 3 IBV vaccine types and at approximately the same titer for Ark and Mass, it appears that something else must be contributing to the poor efficacy of the Ark vaccine when given by hatchery spray cabinet. In our studies, we found little or no circulating antibodies against IBV by ELISA at 5 days post-challenge. It has been reported that low levels of humoral antibodies did not always indicate a lack of protection against IBV, and that mucosal IgA in the upper-respiratory tract plays an important role in preventing infection (13, 21, 26). We did not examine mucosal IgA.

In experiment 2, we examined either a 1X or 2X dose given to groups of either 20 or 40 birds and found that only the group of 20 birds vaccinated with 40 doses of Ark vaccine showed better protection and detectable levels of vaccine virus at 28 days of age compared to the other groups. It is not clear why only this group had evidence of vaccine virus replication, but it is

possible that delivering a 2X dose in 7ml (0.35ml/bird) is a critical combination of dose and volume compared to groups of 40 birds sprayed with a 2X dose in 7ml (0.175ml/bird) or birds that received a 1x dose. It should also be realized that delivering a 2X dose by spray likely does not equal the same dose delivered by eye-drop.

Selection of subpopulations has been described for the IBV Ark type vaccines after only one passage in chickens (23, 29). In experiment 3, we isolated an Ark vaccine virus at the peak titer in broilers (21 days post-vaccination), passaged it one time in embryonated eggs to increase the titer and designated it Ark-RI-EP1. Assuming that Ark-RI-EP1 would be more fit to replicate in birds than the original Ark vaccine, we gave the virus to birds by eye drop and found that the replication pattern was nearly identical to the original Ark vaccine with a peak titer of the viruses in the trachea at 3 days of age and declining to undetectable levels by 21 days post-vaccination. This data was in contrast to Ark vaccine administrated using a hatchery spray cabinet where the virus was not detected until 21 days post-vaccination, presumably because of the route of inoculation.

To further examine the influence that the route of inoculation has on the dynamics of replication in the birds, we examined 3 different routes of vaccination using both the Ark vaccine and Ark-RI-EP1 virus in experiment 4. Similar to the previous experiment, Ark vaccine and Ark-RI-EP1 were detected within the first week after vaccination when the viruses were administrated by eye drop. However, spray vaccination showed a delay (>3 weeks) in detecting both the Ark vaccine and Ark-RI-EP1 virus in the birds. Thus, it appears that the Ark-RI-EP1 virus is not more fit than the original Ark vaccine for infection and replication in broilers when administered by hatchery spray cabinet. When the viruses were given by drinking water, the Ark vaccine virus was undetectable out to 35 days of age, whereas the Ark-RI-EP1 virus had a peak

titer at 7 days of age. This observation suggests that there are differences between the Ark vaccine and the Ark-RI-EP1 with regard to infection and/or replication in the birds. This was further reinforced by the greater level of protection observed in birds given the Ark-RI-EP1 vaccine in the drinking water.

To further examine differences between the Ark vaccine and the Ark-RI-EP1 virus we examined the S1 gene and the nsp 3 gene sequences. S1 gene analysis showed 9 non-synonymous differences between the Ark vaccine and the Ark-RI-EP1 consensus sequences. In addition, there was a 3-nucleotide deletion resulting in the loss of asparagine (Asn) at residue 345 in Ark-RI-EP1. Compared to all previously published subpopulations of Ark vaccines (12, 22, 27), Ark-RI-EP1 is very similar to C3 (NCBI accession number: EU359626) (12), but has 2 additional mutations at nucleotide position 188 (C->T) and 233 (C->T). Ark-RI-EP1 shares 3 amino acid changes (Tyr43His, Ser213Ala, Tyr326Asn) and the loss of asparagine (Asn) at amino acid position 345 with previously published vaccine viruses EU283047, EU283051, EU283053 and EU283056 (22). In this previous study, reisolated Ark vaccines from different manufacturers were compared and it was indicated that minor double peaks existed at these 3 positions in original vaccines and a deletion of Asn345 was observed in all reisolated Ark vaccine viruses. As noted by McKinley et al. (22), it is possible that these changes are the result of selection of a more fit subpopulation and a deletion of 3 nucleotides coding for Asn345 could be important for *in-vivo* replication of Ark type vaccine. In the previous studies, the reisolated vaccine viruses were collected up until 14-days post-vaccination. The differences between those viruses and our reisolated vaccine virus indicate that a different major subpopulation of the Ark vaccine was selected in the birds by 21 days post-vaccination, suggesting that subpopulations may be changing during the course of an infection.

It has been reported that nonstructural replicase genes are associated with the pathogenicity of infectious bronchitis virus (2, 3) and many changes, especially in the nsp 3 gene, have been reported (25). The nsp 3 gene sequence of Ark-RI-EP1 showed one non-synonymous mutation resulting in a glycine to aspartic acid change, when compared to the Ark vaccine virus. Only one amino acid sequence change between Ark-RI-EP1 and the Ark vaccine in the nsp 3 gene suggests that the attenuation of Ark-RI-EP1 was maintained, which is what we observed in the vaccinated birds.

In conclusion, it appears that hatchery spray cabinet vaccination of broilers with Ark type vaccine is not sufficient to immunize birds against homologous Ark-DPI challenge virus, although eye drop vaccination with that same vaccine and the same dose did induce protective immunity. This indicates that the vaccine virus is immunogenic. Hatchery spray cabinet vaccinators typically deliver 100 doses of vaccine to 100 birds in a total of 7mls. We found that a 2X dose of the Ark vaccine in a total of 7mls given to 20 birds was sufficient to induce some protection whereas a 2X dose in 7mls given to 40 birds was not, suggesting that the combination of dose and vaccine volume are important. Finally, an isolate of Ark vaccine (Ark-RI-EP1) from a broiler at 21 days of age may have been more fit to infect and replicate in the birds, although we saw no differences in infection, replication or protection as compared to the original Ark vaccine when delivered by hatchery spray cabinet. Clearly the efficacy of Ark type IBV vaccines when delivered by hatchery spray cabinet is not acceptable and the key to protection against Ark and perhaps reducing vaccine persistence in the field lies in developing a sound method of immunization in the hatchery.

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Table 5-1. IBV real-time RT-PCR mean C_T value ± S.E.M and histopathology score for experiment 1 at 5 days post challenge.

Group	Vaccine	Challenge	Real-time RT-PCR				Histopath score[C]
			Tracheal or choanal swab (positive[A]/total)	Mean C_T Value[B] (± S.E.M)	Tear (positive/total)	Mean C_T Value (± S.E.M)	
Control group	None	None	0/5	Neg[C]	0/5	Neg	1.9
	Ark	None	0/4	Neg	2/4	33.87 ± 0.65	2.1
	Mass	None	0/5	Neg	0/5	Neg	1.8
	GA98	None	1/5	33.82	5/5	33.05 ± 0.76	2
	Ark& Mass	None	0/5	Neg	1/5	34.18	1.7
	Ark & GA98	None	2/5	32.47 ± 0.98	0/5	Neg	2
	None	Ark-DPI	5/5	22.87 ± 0.11	5/5	16.80 ± 0.77	2.5 *
	None	Mass 41	5/5	26.40 ± 0.50	5/5	15.45 ± 0.58	2.9 *
	None	GA98	5/5	24.94 ± 0.45	5/5	18.01 ± 0.53	3 *
Experimental group	Ark	Ark-DPI	5/5	24.96 ± 1.83	5/5	18.28 ± 1.88	2.7 *
	Mass	Mass41	4/5	30.93 ± 1.39	5/5	30.18 ± 0.51	2.2
	GA98	GA98	1/5	31.773	5/5	31.83 ± 0.69	2
	Ark & Mass	Ark-DPI	5/5	27.05 ± 0.22	4/5	22.64 ± 1.67	2.5 *
	Ark & Mass	Mass 41	1/5	31.32	5/5	30.62 ± 0.36	2.1
	Ark & GA98	Ark-DPI	5/5	28.78 ± 0.54	5/5	20.88 ± 0.85	2.7 *
	Ark & GA98	GA98	2/5	34.18 ± 0.19	5/5	32.64 ± 0.57	2

[A]Positive: a sample with C_T value over limit of detection (C_T value < 34.95) was considered as a positive.

[B]Mean C_T values were calculated only from the positive samples. Larger numbers indicate less viral RNA present in the sample.

[C]Histopathologic score: 1= normal, 2= focal, 3= multifocal, 4= diffuse

[D]Neg = below limit of detection (C_T value > 34.95).

* indicates significantly different value compare to negative control ($P < 0.05$).

Table 5-2. Clinical sign, IBV real-time RT-PCR mean C_T value ± S.E.M for experiment 2 at 5 days post challenge.

Group	Vaccine (dose)	Challenge	Clinical sign	Real-time RT-PCR			
				Tracheal or choanal swab (positive[A]/total)	Mean C_T Value[B] (± S.E.M)	Tear (positive/total)	Mean C_T Value (± S.E.M)
Negative control	None	None	0/5	0/5	Neg[C]	0/5	Neg
Challenge control	None	Ark-DPI	8/10	10/10	28.88 ± 0.58	10/10	20.47 ± 0.67
20 birds/20 doses	Ark (20)	Ark-DPI	8/10	10/10	28.46 ± 0.28	10/10	19.42 ± 0.37
20 birds/40 doses	Ark (40)	Ark-DPI	2/10	8/10	31.22 ± 1.26	10/10	22.94 ± 0.37
40 birds/40 doses	Ark (40)	Ark-DPI	10/10	10/10	29.50 ± 0.38	10/10	19.60 ± 0.40
40 birds/80 doses	Ark (80)	Ark-DPI	10/10	10/10	29.73 ± 0.69	10/10	19.47 ± 0.91

[A]Positive: a sample with C_T value over limit of detection (C_T value < 34.59) was considered as a positive.

[B]Mean C_T values were calculated only from the positive samples. Larger numbers indicate less viral RNA present in the sample.

[C]Neg = below limit of detection (C_T value > 34.95).

Table 5-3. Clinical sign, IBV real-time RT-PCR mean C_T values ± S.E.M, and histopathology score for experiment 4 at 5 days post challenge.

Group	Vaccine	Challenge	Clinical sign	Real-time RT-PCR				Histopath score[C]
				Tracheal or choanal swab (positive[A]/total)	Mean C_T Value[B] (± S.E.M)	Tear (positive/total)	Mean C_T Value (± S.E.M)	
Control group	None	None	0/5	0/5	Neg[F]	0/5	Neg	1.66
	Ark (spray)	None	0/5	0/5	Neg	0/5	Neg	1.8
	Ark (D.W[D])	None	0/5	1/5	30.30	1/5	28.10	2
	Ark (eye[E])	None	0/5	0/5	Neg	0/5	Neg	2.2
	None	Ark-DPI	9/10	10/10	25.87 ± 0.26	10/10	19.05 ± 1.04	2.9 *
Experimental group	Ark (spray)	Ark-DPI	9/10	10/10	26.70 ± 0.30	10/10	19.12 ± 1.04	3 *
	Ark (D.W)	Ark-DPI	8/10	10/10	26.34 ± 0.86	10/10	20.69 ± 1.75	2.8 *
	Ark (eye)	Ark-DPI	1/10	8/10	31.22 ± 1.26	10/10	27.84 ± 5.73	2.4
Control group	None	None	0/5	0/5	Neg	0/5	Neg	1.7
	Ark-RI-EP1(spray)	None	0/5	0/5	Neg	0/5	Neg	1.8
	Ark-RI-EP1(D.W)	None	0/5	1/5	34.15	0/5	Neg	1.7
	Ark-RI-EP1 (eye)	None	0/5	1/5	34.66	0/5	Neg	1.8
	None	Ark-DPI	8/10	10/10	27.42 ± 0.47	10/10	19.31 ± 0.42	3 *
Experimental group	Ark-RI-EP1 (spray)	Ark-DPI	8/10	10/10	26.11 ± 0.33	10/10	19.29 ± 0.38	3 *
	Ark-RI-EP1 (D.W)	Ark-DPI	2/10	1/10	30.94	6/10	31.63 ± 1.61	1.9
	Ark-RI-EP1 (eye)	Ark-DPI	0/10	4/10	30.09 ± 0.68	4/10	32.03 ± 0.93	2.1

[A]Positive: a sample with C_T value over limit of detection (C_T value < 34.95) was considered as a positive.

[B]Mean C_T values were calculated only from the positive samples. Larger numbers indicate less viral RNA present in the sample.

[C]Histopathologic score: 1= normal, 2= focal, 3= multifocal, 4= diffuse

[D]D.W. = Drinking Water

[E]eye = Eye drop

[F]Neg = below limit of detection (C_T value > 34.95).

* indicates significantly different value compare to negative control ($P < 0.05$).

Table 5-3. continue

Table 5-4. Difference in S1 gene and nsp3 gene of parental commercial Ark-DPI vaccine and Ark-RI-EP1.

Virus		S1										nsp3[A]
	Nucleotide position	127	188	226	233	355	511	593	637	976	1033-1035	3758[B]
	Amino acid position	43	63	76	78	119	171	198	213	326	345	1253[B]
Ark-DPI [C]	Nucleotide	T	C	C	C	T	T	A	T	T	AAT	G
	Amino acid	Tyr	Pro	Leu	Ala	Ser	Tyr	Lys	Ser	Tyr	Asn	Gly
Ark-RI-EP1	Nucleotide	C	T	T	T	C	C	C	G	A	–[D]	A
	Amino acid	His	Leu	Phe	Val	Pro	His	Thr	Ala	Asn	–	Asp
C3[E]	Nucleotide	C	C	T	C	C	C	C	G	A	–	N/A[F]
	Amino acid	His	Pro	Phe	Ala	Pro	His	Thr	Ala	Asn	–	N/A

[A]nsp3 = nonstructural protein 3 in open reading frame (ORF) 1ab

[B]Nucleotide position and amino acid position is based on ORF1ab from Arkansas DPI (NCBI accession number GQ504720). NSP3 position is ORF 1ab is 679G – 2256G

[C]Ark-DPI = Ark-DPI vaccine

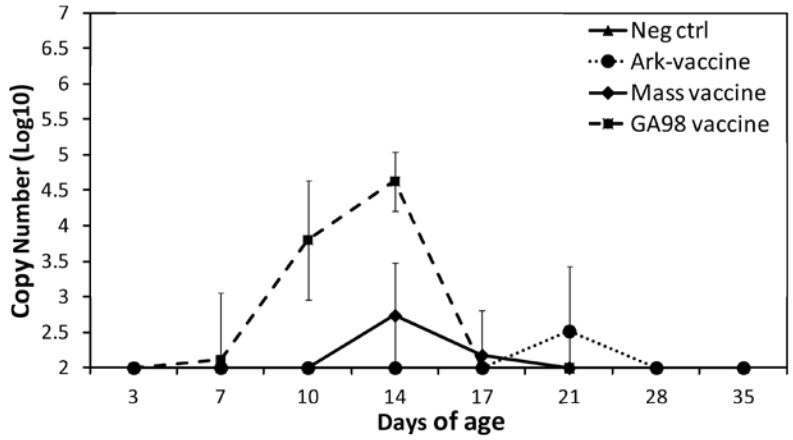
[D] – = deletion

[E]C3= GeneBank accession EU359626 (Gallardo et al., 2010(13).

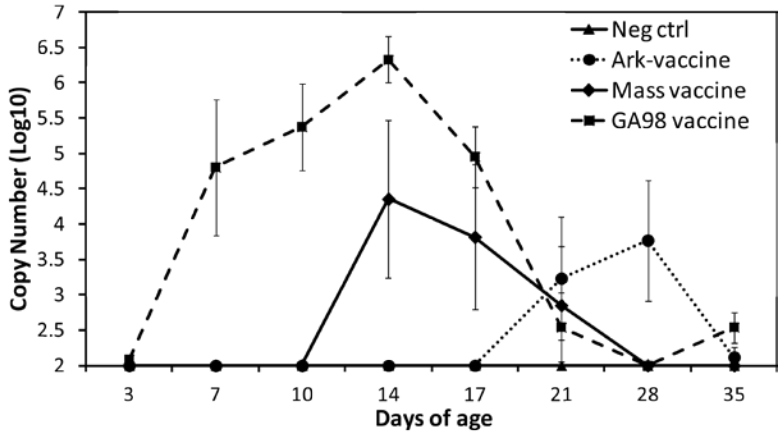
[F]N/A= Not available

Figure 5-1. Experiment 1. Level of vaccine virus in broilers vaccinated at one-day of age with a hatchery spray cabinet. Tracheal swabs and tears from five birds were taken at each time point and tested individually by real-time RT-PCR using a universal IBV probe (IBV-5'G). All tracheal swabs and tears at each time point were collected from vaccinated and non-challenged birds from each vaccine group. Level of virus is shown in genome copy number (Log10). All samples were tested in triplicate. (A) Tracheal swabs taken from birds vaccinated with single vaccine type (Ark vaccine, Mass vaccine, GA98 vaccine). (B) Tracheal swabs from vaccinated birds with combined vaccines (Ark and Mass vaccine, Ark and GA98 vaccine).(C) Tears taken from birds vaccinated with single vaccine. (D) Tears from vaccinated birds with combined vaccines. Values below limit of detection ($<10^2$) are not shown.

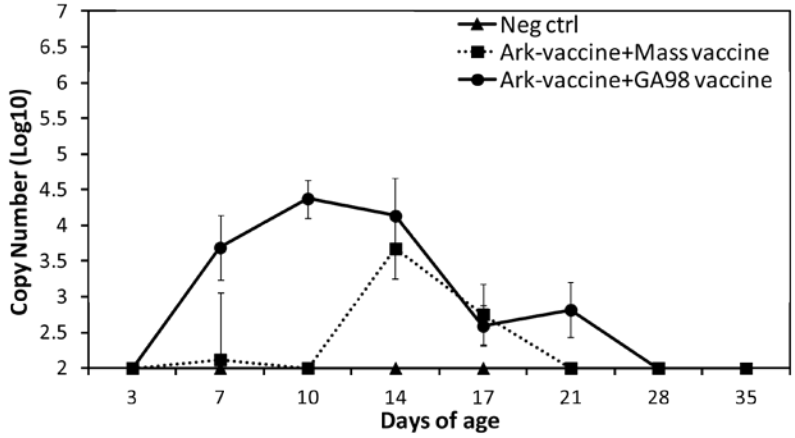
A. Tracheal swabs



C. Tears



B. Tracheal swabs



D. Tears

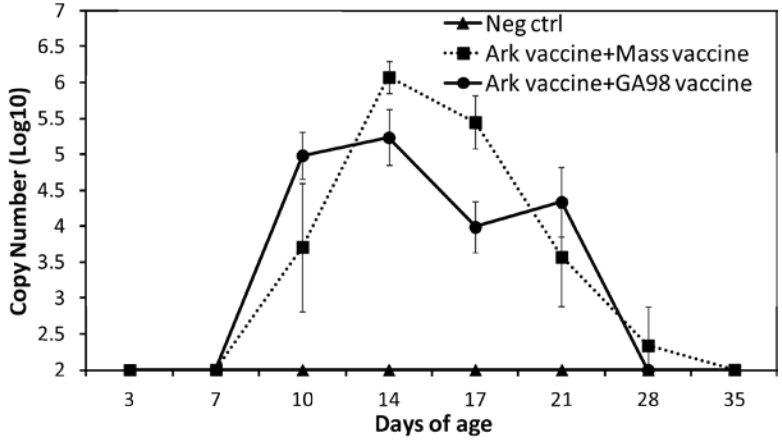
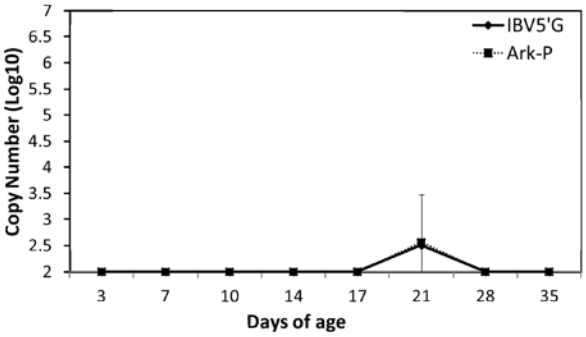
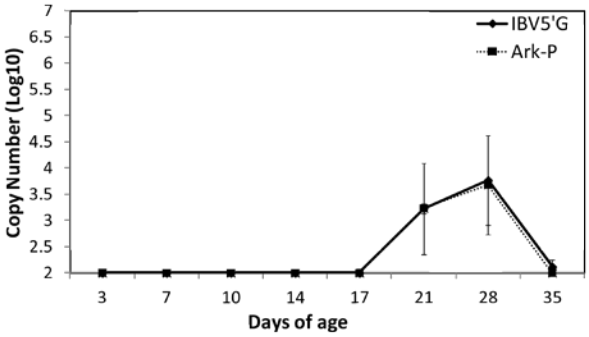


Figure 5-2. Experiment 1. Amount of Ark vaccine virus in birds vaccinated with combined vaccines. Tracheal swabs and tears obtained from five birds at each time point were analyzed by real-time RT-PCR using a universal IBV probe (IBV5'G) and Ark-specific probe (Ark-P). (A) Tracheal swabs from birds vaccinated with Ark vaccine only. (B) Tracheal swabs from Ark and Mass combined vaccine group. (C) Tracheal swabs from Ark and GA98 combined vaccine group. (D) Tears from birds vaccinated with Ark vaccine only. (E) Tears from Ark and Mass combined vaccine group. (F) Tears from Ark and GA98 combined vaccine group. Values below limit of detection ($<10^2$) are not shown.

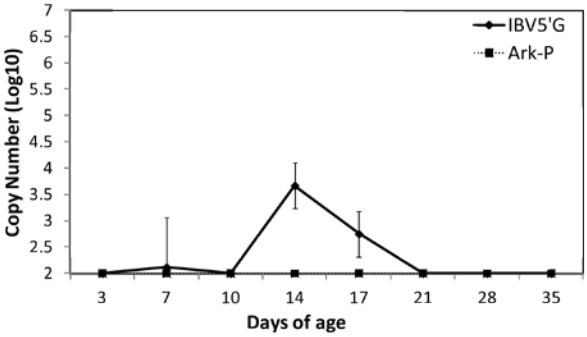
A. Tracheal swab



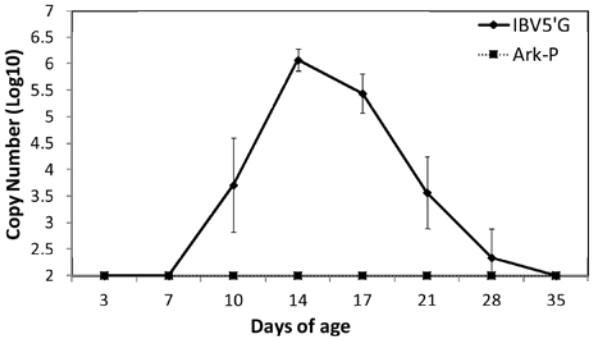
D. Tear



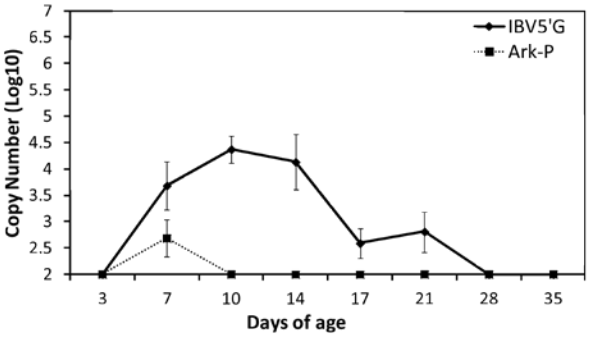
B. Tracheal swab



E. Tear



C. Tracheal swab



F. Tear

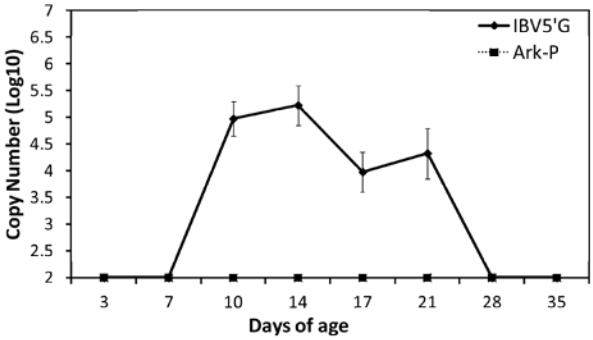
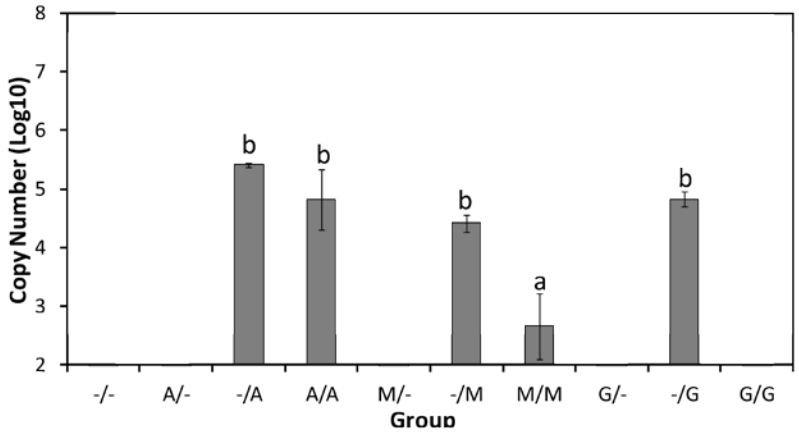
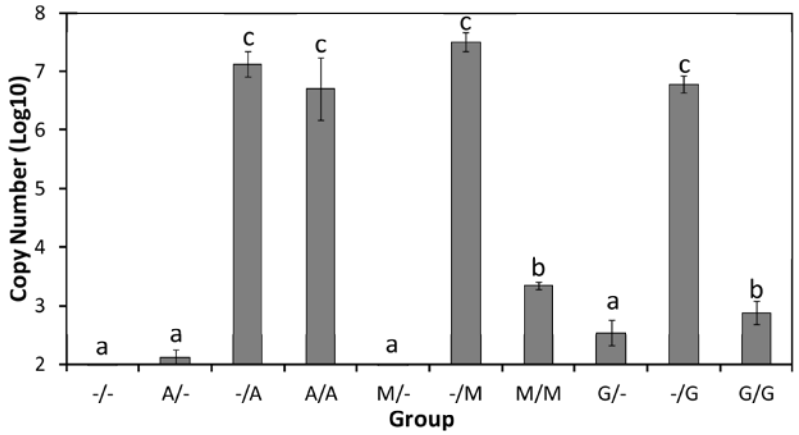


Figure 5-3. Experiment 1. Level of clearance of challenge virus in vaccinated birds. Amount of challenge virus detected in the birds is shown in genome copy number (Log10). Mean genome copy numbers (Log10) were calculated using all sample values. (A) Tracheal swabs from single vaccine groups. (B) Tracheal swabs from combined vaccine groups. (C) Tears from single vaccine groups. (D) Tears from combined vaccine groups. Vaccine virus/ Challenge virus (− = nothing given). A = Ark, M = Mass, G = GA98. Groups not sharing the same alphabet letter indicate significant differences ($p<0.05$). Level of virus is shown in genome copy number (Log10). Error bars indicate mean copy numbers (Log10) ± S.E.M. Values below limit of detection ($<10^2$) are not shown.

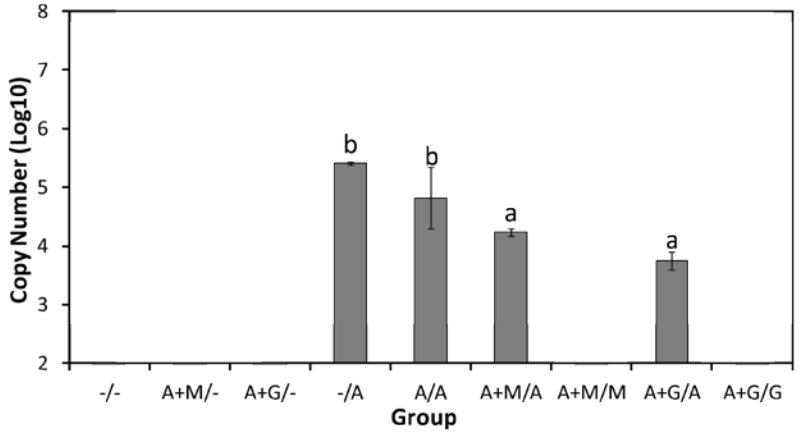
A. Tracheal swabs



C. Tear



B. Tracheal swabs



D. Tear

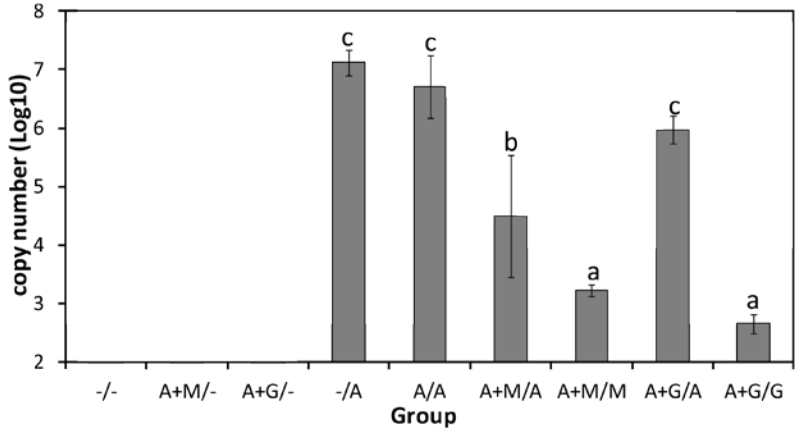


Figure 5-4. Experiment 3. Level of virus in the tracheas of the birds is shown in genome copy number (Log10) after vaccination with Ark vaccine, or Ark-RI-EP1 by eye drop. Choanal swabs were taken from 10 birds at each time point and analyzed by real-time RT-PCR. Ark-RI-EP1=reisolated Ark vaccine passed 1 time in embryonated eggs. Values below limit of detection ($<10^2$) are not shown.

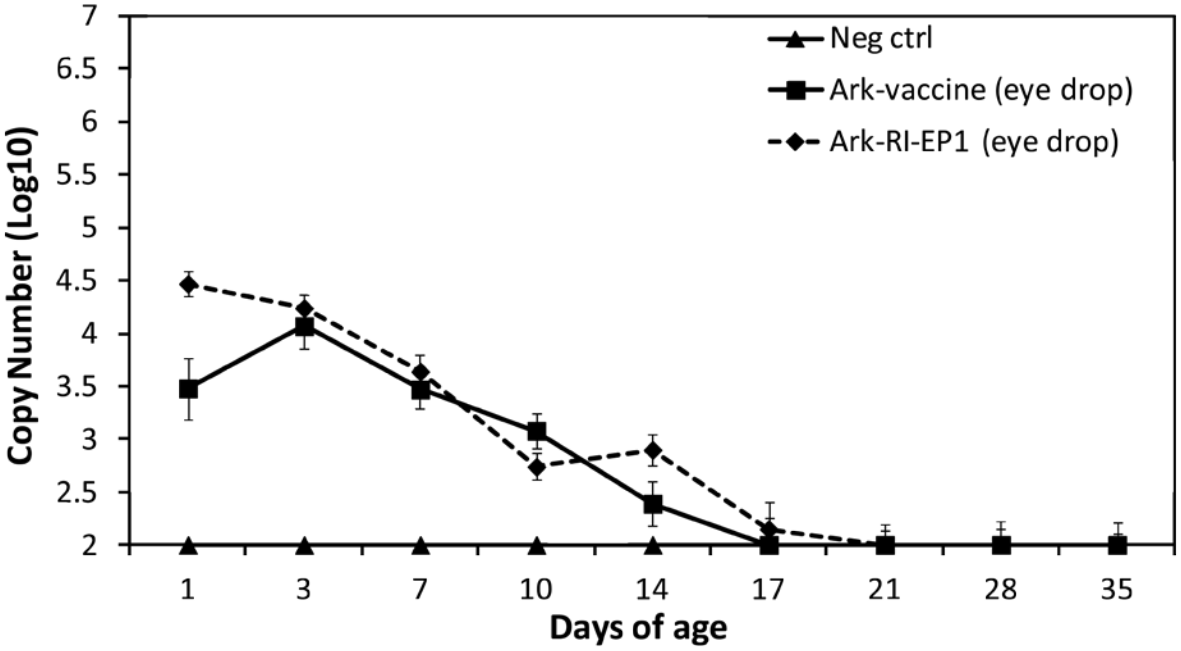
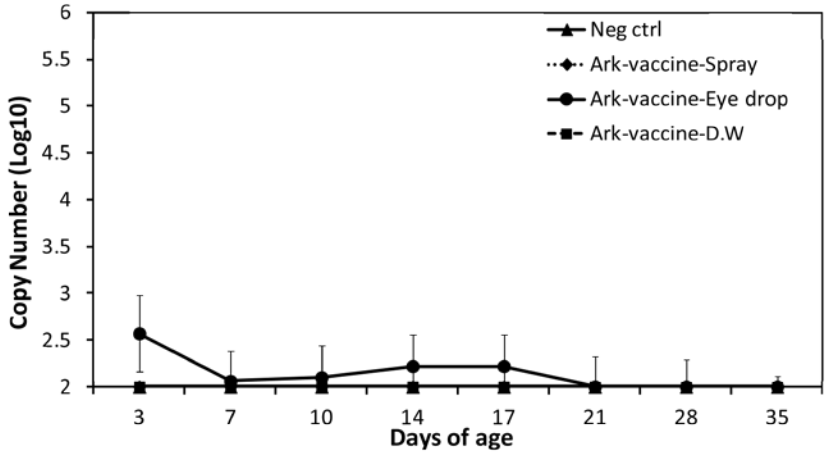
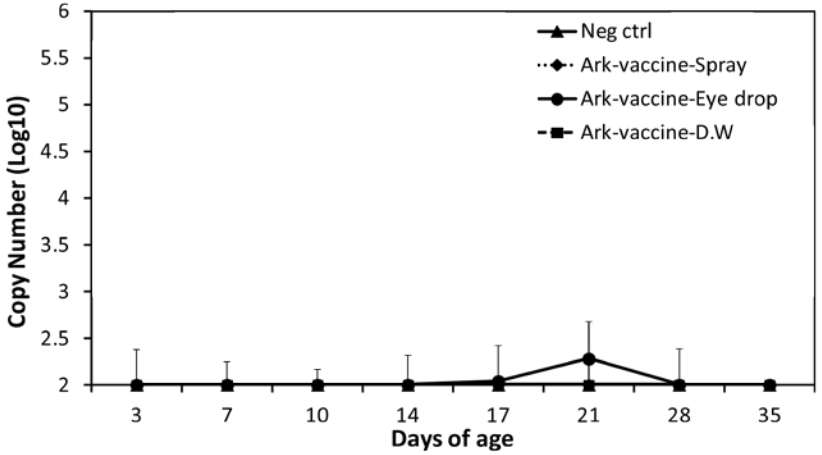

Neg ctrl
Ark-vaccine (eye drop)
Ark-RI-EP1 (eye drop)
Copy Number (Log10)
7
6.5
6
5.5
5
4.5
4
3.5
3
2.5
2
1
3
7
10
14
17
21
28
35
Days of age

Figure 5-5. Experiment 4. Level of virus in the birds vaccinated with Ark vaccine or Ark-RI-EP1 is shown in genome copy number (Log10). Mean genome copy number (Log10) was calculated using all sample values. (A) Choanal swabs from birds vaccinated with Ark-vaccine by spray (Ark-vaccine-spray), eye-drop (Ark-vaccine-Eye drop), or via drinking water (Ark-vaccine-D.W). (B) Choanal swabs from birds vaccinated with Ark-RI-EP1 by spray (Ark-RI-EP1-spray), eye-drop (Ark-RI-EP1-Eye drop), or via drinking water (Ark-RI-EP1-D.W). (C) Tears from birds vaccinated with Ark-vaccine. (D) Tears from birds vaccinated with Ark-RI-EP1. Error bars indicate mean genome copy number (Log10) ± S.E.M. Values below limit of detection ($<10^2$) are not shown.

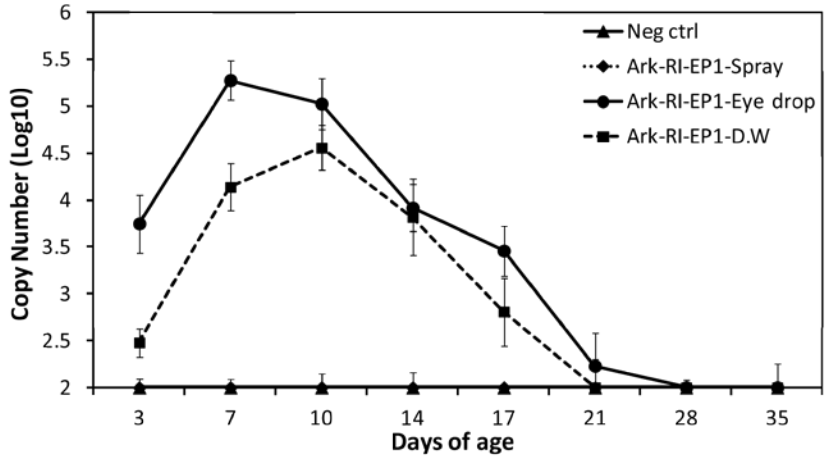
A. Choanal swabs



C. Tears



B. Choanal swabs



D. Tears

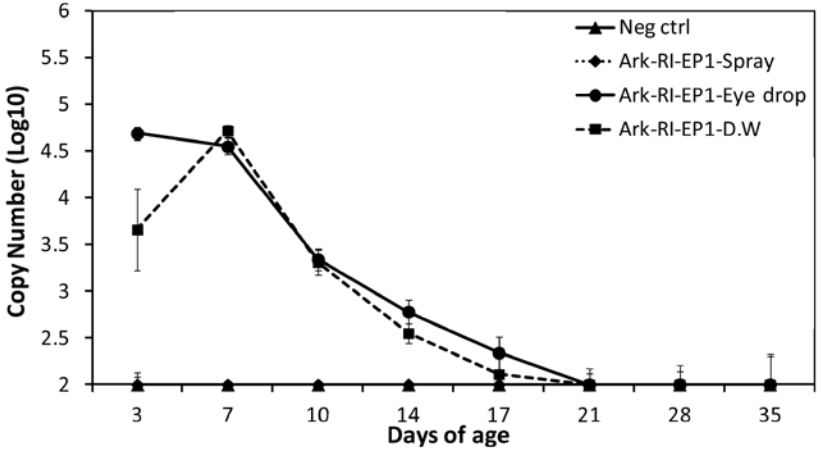
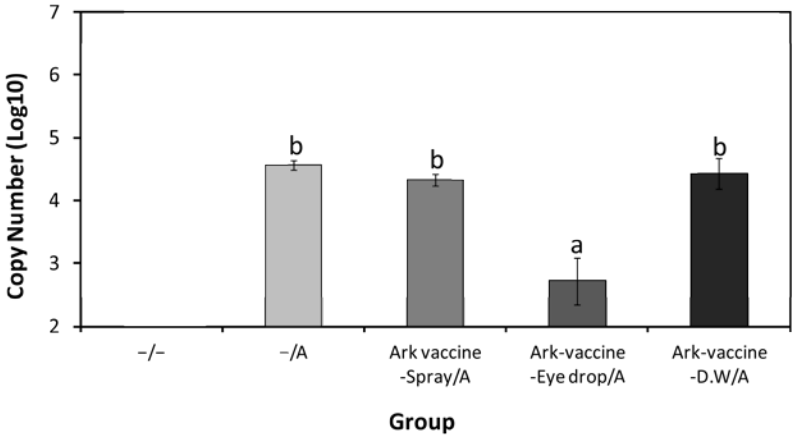
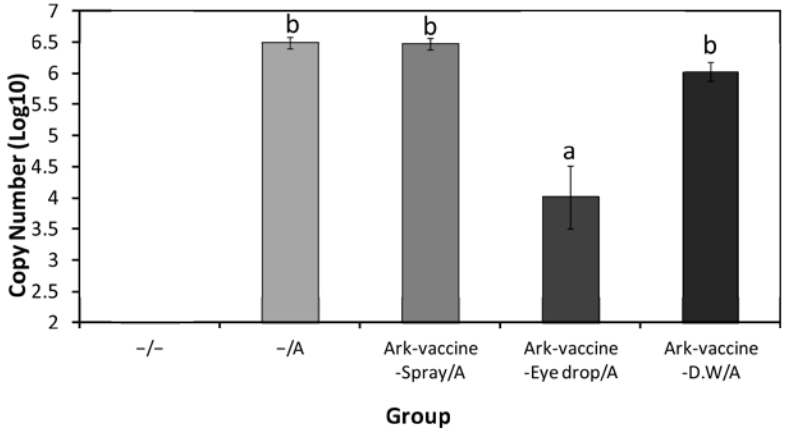


Figure 5-6. Experiment 4. The amount of challenge virus detected in the birds is shown as genome copy number (Log10). (A) Choanal swabs from birds vaccinated with Ark-vaccine. (B) Choanal swabs from birds vaccinated with Ark-RI-EP1. (C) Tears from birds vaccinated with Ark-vaccine. (D) Tears from birds vaccinated with Ark-RI-EP1. Vaccine virus/ Challenge virus (− = nothing given). A= Ark-DPI challenge virus, Ark-vaccine = Ark-DPI commercial vaccine, Ark-RI-EP1= reisolated Ark vaccine passed 1 time in embryonated eggs. Spray= spray vaccination, Eye-drop= eye drop vaccination, D.W= vaccination via drinking water. Groups not sharing the same alphabet letter indicate significant differences ($p<0.05$). Error bars indicate mean genome copy number (Log10) ± S.E.M. Values below limit of detection ($<10^2$) are not shown.

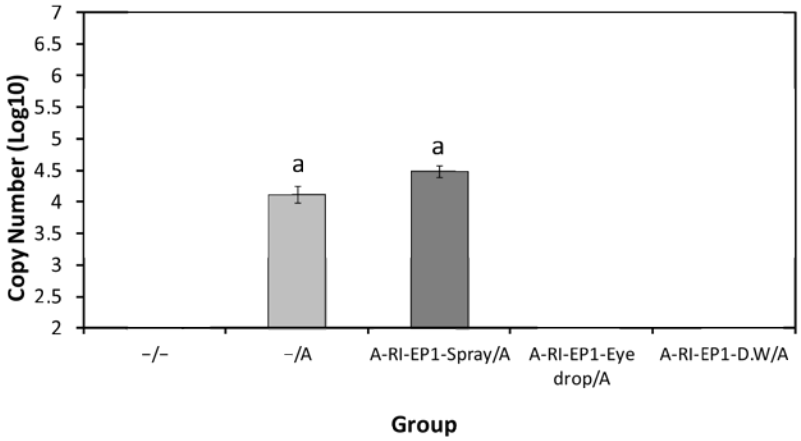
A. Choanal swabs



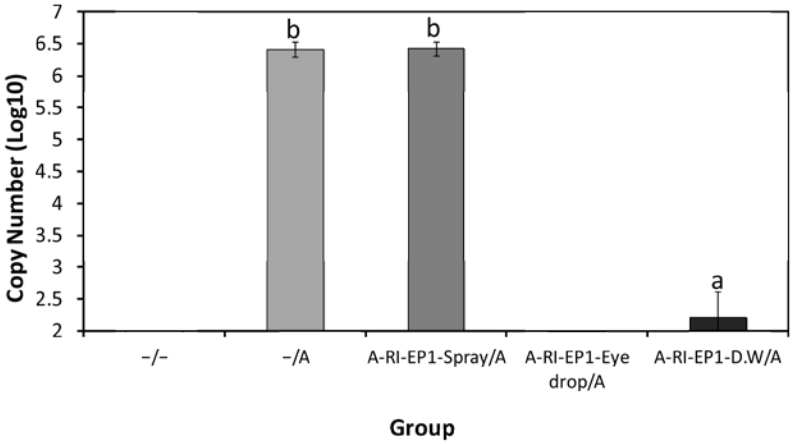
C. Tears



B. Choanal swabs



D. Tears



CHAPTER 6

MORPHOLOGICAL DIFFERENCES BETWEEN AVIAN CORONAVIRUS INFECTIOUS BRONCHITIS VIRUS ARK-DPI AND MASS TYPE COMMERCIAL VACCINES OBSERVED WITH AN ELECTRON MICROSCOPE[4]

[4]Roh,H-J. B.J.Jordan, D.A. Hilt, and M.W.Jackwood. Submitted to *Avian Diseases*.

Abstract

Commercially available Ark-DPI vaccine was evaluated to understand its poor replication in birds after spray application. Our hypothesis was the potential mechanical damage on vaccine viruses by spray application would result in failure of replication. Vaccine back titration after application showed there was no significant titer change in Ark-DPI vaccine after spray. Concordantly, neither Ark-DPI nor Mass vaccine showed morphological differences in pre or post spray samples in electron micrographs. Rather, in all Ark-DPI vaccine samples, virus particles with few or no intact spikes were more frequently found compared to the Mass vaccines.

Key word: Ark-DPI, electron microscope, infectious bronchitis virus, morphology, vaccine

Introduction

Infectious bronchitis virus (IBV) is the causative agent of infectious bronchitis (IB), a highly contagious upper respiratory disease of chickens. The disease affects productivity of chickens by reducing feed conversion rate in broilers and decreasing egg production and egg quality in layers. Vaccination with live attenuated IBV strains is the most routinely applied method to protect birds against pathogenic IBV strains in the broiler industry. Replication of live attenuated vaccine strains induces a local, mucosal immune response that protects birds from infection.

In the field, vaccine is often applied in two stages; a hatchery vaccination at day 1 for initial priming of the immune response and a field vaccination at approximately 14 days of age, which is designed to boost the local immune response and protect the birds for the length of the growout. Currently, Arkansas-DPI (Ark-DPI), Massachusetts (Mass) and Georgia (GA98) are the most frequently used vaccine strains in the U.S, and adequately vaccinated chickens should be protected against the homologous pathogenic field strains. However, there have been reports indicating that the Ark virus can persist in vaccinated birds (3, 4) and Ark-DPI-like viruses have emergenced in the field (6). In addition, Ark-DPI vaccinated birds brought from the field and challenged in an experimental setting with pathogenic Ark virus were not sufficiently protected (5). Experiments in our laboratory confirmed that Ark-DPI vaccine failed to provide adequate protection against homologous challenge when the vaccine was applied using a hatchery spray cabinet, whereas Mass and GA98 type vaccines applied in the same manner, successfully protected birds against homologous challenge virus (7). In that experiment, we also found that Ark-DPI vaccine applied by eye drop replicated to relatively high levels and was efficacious,

whereas Ark-DPI vaccines applied by the hatchery spray cabinet were replicating poorly in the vaccinated chickens.

In an attempt to elucidate why Ark-DPI vaccines are not efficacious when applied by a hatchery spray cabinet, we used an electron microscope to examine the morphological characteristics of that vaccine virus prior to and after spray and compared it to Mass type vaccine. In addition, the Ark-DPI vaccine titer prior to and after spray was examined in embryonated eggs and the virus was tested for replication in chickens.

Materials and Methods

Vaccines

Commercially available monovalent live attenuated Ark-DPI and Mass type vaccines were used in this study.

Chickens

Commercial non-vaccinated broiler chicks were obtained from a commercial source at 1 day of age and 1 day-old specific pathogen free (SPF) chicks were obtained from Sunrise Farms Inc. (Catskill, NY). All chicks were maintained in positive-pressure Horsfal isolation units at the Poultry Diagnostic and Research Center (University of Georgia, Athens GA). Feed and water were provided *ad libitum*.

Vaccine replication experiment

To examine replication of Ark-DPI vaccine *in vivo*, we vaccinated 1-day-old chicks using a commercial hatchery spray cabinet and evaluated replication of vaccine virus in the upper-respiratory tract. The Mass vaccine previously shown to replicate efficiently and stimulate an adequate immune response in chicks was used as a positive control. Briefly, each vaccine stock

from the manufacturer was rehydrated in PBS (1000 doses/ml) and working solutions that contained 100 vaccine doses in a total volume of 7ml were prepared. The working solutions were back tittered in embryonated eggs and the titer was 1 X $10^{4.85}$ 50% egg infectious doses (EID_{50})/ml for Ark-DPI vaccine and 1 X $10^{4.5}$ EID_{50}/ml for Mass vaccine. Each vaccinated group of birds consisted of five 1-day-old SPF chicks and five 1-day-old broiler chicks. A single application of 7ml working vaccine solution was applied to the chicks using a commercial hatchery spray cabinet. Birds in a same vaccine group were kept together in a single isolator and choanal swabs were taken from all birds at day 7 and day 10 post vaccination (p.v).

Real-time RT-PCR

Viral RNA was extracted from choanal swabs using the MagMAX-96 RNA Isolation Kit (Ambion Inc., Austin TX) according to the manufacturer's protocol on a KingFisher magnetic particle processor (Thermo Scientific, Waltham, MA). The real-time RT-PCR test was performed using a Smart Cycler II (Cepheid, Sunnyvale, CA) and the AgPath-ID™ One-Step RT-PCR kit (Ambion Inc.) according to the manufacturer's recommendations. Primers, probe and real-time RT PCR conditions used in this study were previously described (1). The relative amount of virus was expressed as the cycle threshold (C_T) value.

Vaccine titration

To determine if there is a drop in Ark-DPI vaccine titer after spray vaccination, which could potentially affect vaccine replication efficiency, the Ark-DPI working solution was collected before and after spray application. Samples were collected from the Mass vaccine working solution in the same manner. The samples were titrated using embryonated SPF chicken eggs and EID_{50} was calculated by the method of Reed and Muench.

Transmission electron microscope

The working vaccine solution samples collected before and after spray application for both Ark-DPI and Mass vaccines were also observed using a transmission electron microscope to identify any potential mechanical damage to virus particles caused during spray application. Briefly, each sample was stained with 3% aqueous phosphotungstic acid (PTA) pH 7.0 for 30 sec on a formvar carbon-coated grid and viewed with a JEM-1210 transmission electron microscope (JEOL, Inc., Tokyo, Japan). For each sample, 3 grids were prepared and observed.

Results and Discussion

We hypothesized that Ark vaccine might be mechanically damaged during spray application, therefore causing it to fail to replicate in birds. To test this hypothesis, we spray vaccinated chicks with Ark-DPI vaccine and monitored replication of the vaccine. The Mass vaccine was used as a positive control. To rule out maternal antibody interference of vaccine viruse replication, SPF chicks were also vaccinated. The real-time RT-PCR data for choanal swabs are shown in Table 6-1. As expected, chicks vaccinated with Mass vaccine were positive for vaccine viruses at both day 7 and day 10 p.v. At day 7 p.v, 4 SPF chicks out of 5 and 2 out of 5 broiler chicks were positive for Mass virus and by day 10 p.v, all 5 of the SPF chicks and 4 of 5 broilers were positive with average C_T values of 28.46 and 31.51 respectively. On the other hand, in the Ark-DPI vaccinated group, only 2 out of 5 SPF birds were positive at day 7, with an average C_T value of 37.92, and by day 10 p.v, no SPF birds were positive for vaccine viruses. Similarly, Ark-DPI vaccine virus failed to replicate in broilers (0 out of 5) at day 7 p.v and only one broiler was vaccine virus positive (C_T value: 37.42) at day 10 p.v. This experiment confirmed inefficient replication of hatchery cabinet sprayed Ark-DPI vaccine viruses in chicks.

To determine if a significant titer drop occurred in Ark-DPI vaccine, we examined the titer of vaccine working solution samples collected before and after spray application. The Mass vaccine was also tested as a control. As shown in Table 6-2, there was only a half $\log_{10}$ decrease in Ark-DPI vaccine titer after spray. A similar titer drop was observed in the Mass vaccine sample obtained after spray, however; the Mass vaccine titer after spray was almost a full $\log_{10}$ higher than the Ark-DPI vaccine. It is not clear why the Ark-DPI vaccine can infect and replicate in an embryonated egg and not in a 1-day old chick, but it is possible that more infectious virus is needed to infect a chick then is needed to infect an embryonated egg.

The spike glycoproteins on the surface of the virus, which are involved in attachment and infection of the host cell, can be removed from the virus by high speed centrifugation (100,000g), incubation at 37ºC or exposure to urea (2). We considered the possibility that Ark-DPI vaccine virus could be losing spikes due to mechanical sheering forces during spray vaccination. To examine potential morphological changes in the vaccine viruses, working solutions of each vaccine collected before and after spray were observed using a transmission electron microscope (Fig. 6-1). We found no significant morphological differences in virus particles before or after spray for either the Ark-DPI vaccine or the Mass vaccine. Therefore it appears that spray vaccination itself does not cause any observable mechanical damage to vaccine viruses. However, interestingly, in the Ark-DPI vaccine samples, a majority of the virus particles viewed both prior to and after spray had few to no intact spikes. Some Ark-DPI vaccine virus particles with intact spikes were found in these samples but at a much lower frequency than virus particles with few or no spikes. As a control, Mass vaccine samples collected before and after spray were also examined. The majority of Mass vaccine virus particles in all the samples had clearly detectable and intact spikes.

Since spikes on IBV are required for attachment to the host cell, it is possible that fewer spikes on the virus surface might affect the overall virus ability to infect and replicate in the bird. It is not clear why the Ark-DPI vaccine virus was still able to replicate in embryonated eggs as demonstrated by little to no drop in virus titer following spray. And, it is not clear why Ark-DPI vaccine contains fewer intact virus particles and particles with fewer spikes than Mass type vaccine before and after spray. A possible theory is that the serial passage in embryonated eggs resulted in adaptive changes in Ark-DPI vaccine virus such that it is still capable of infecting, entering, and replicating in embryonic cells but less efficient at one of more of those traits in epithelial cells in the upper-respiratory tract of the chick. However it is still not clear why replication of Ark-DPI vaccine in chicks, and efficacy of the vaccine is not affected when it is given by eye-drop.

In conclusion, we confirmed that Ark-DPI vaccine virus was not replicating in vaccinated chicks when it was given using a hatchery spray cabinet while Mass vaccine replication was not affected. Potential mechanical damage by spray application was considered but there were no significant changes in vaccine titration or in observable virus morphology in post spray samples of both Ark-DPI and Mass vaccine. However, the Ark-DPI vaccine had fewer intact virus particles and fewer spikes per virus particle when compared to Mass vaccine samples both before and after spray. The observation that Ark-DPI vaccine can replicate in an embryonated egg and that eye drop administration of Ark-DPI vaccine is efficacious but the same amount of vaccine applied by spray is not, suggests that the amount of infectious virus for the chick is critical and below some threshold of infectivity when applied by hatchery spray cabinet. Further studies are needed to elucidate the cause of these observations.

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Table 6-1. Vaccine viruses detected by real-time RT-PCR from choanal swabs taken from spray vaccinated chicks.

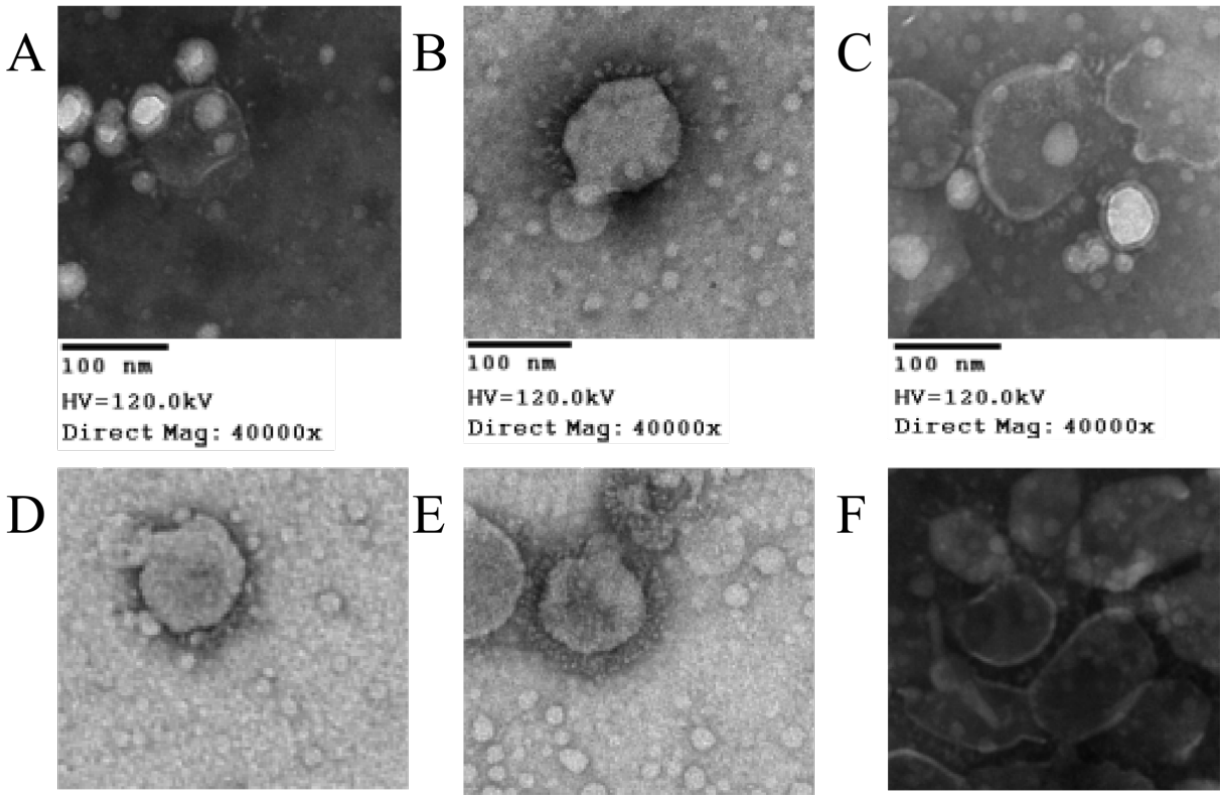
Vaccine	Chicks	Day 7 post vaccination		Day 10 post vaccination	
		Positive birds (+/Total)	Average positive C_T value*	Positive birds (+/ Total)	Average positive C_T value
Mass	Broiler	2/5	28.93	4/5	31.51
	SPF	4/5	31.15	5/5	28.46
Ark-DPI	Broiler	0/5	40	1/5	37.42
	SPF	2/5	37.92	0/5	40

* Only positive values (below C_T value 40) were considered.

Table 6-2. Vaccine titers determined in 10 day of incubation of embryonated eggs before and after spray application.

	Mass vaccine titer	Ark-DPI vaccine titer
Before spray application	$1 \times 10^{4.86}$ EID_{50} /ml	1×10^{4} EID_{50} /ml
After spray application	$1 \times 10^{4.5}$ EID_{50} /ml	$1 \times 10^{3.6}$ EID_{50} /ml

Figure 6-1. Morphology of Ark-DPI (A, B, D and E) and Mass (C and F) vaccine viruses observed with a transmission electron microscope from samples collected before spray application (A-C) and after spray application (D-F). In Ark-DPI vaccine samples, incomplete virus particles (A) and virus particles with few spike proteins (D) were more frequently found than intact virus particles (B, E). Most of virus particles observed in Mass vaccine samples were intact with numerous spike protens (C, F). Samples were negatively stained (Bar = 100nm).


A
100 nm
HV=120.0kV
Direct Mag: 40000x
B
100 nm
HV=120.0kV
Direct Mag: 40000x
C
100 nm
HV=120.0kV
Direct Mag: 40000x
D
E
F

CHAPTER 7

CONCLUSIONS

Avian infectious bronchitis virus is of major concern to the poultry industry worldwide. Its high mutation rate during viral replication results in multiple serotypes and variants, which are antigenically different and generally do not provide cross protection. Therefore, accurate diagnosis in a timely manner and proper vaccination with correct vaccine types are essential keys to control infectious bronchitis outbreaks in the field.

The aims of this project were, first, to develop a diagnostic tool for rapid and accurate detection of IBV and, second, to evaluate the current challenges in field vaccinations with IBV and provide a better understanding of IBV vaccination to improve the current vaccine protocol.

We developed a microsphere-based assay to simultaneously detect and differentiate major U.S serotypes of infectious bronchitis virus. In addition to the microsphere-based assay, a real-time RT-PCR (RRT-PCR) assay was also developed for further utilization in vaccine research. The results showed not only high sensitivity and specificity of the assays but also the ability to detect mixed infections of IBV in a single sample with a potential high throughput application.

To aid the current understanding of Ark-DPI vaccine failures in the field, we evaluated the current coarse-spray hatchery vaccination protocols using mono- and bivalent commercial IBV vaccines. Our data indicate that there was no interference between different serotypes used in hatchery spray vaccinations. In addition, unlike other vaccine types (Mass or GA98), sprayed

Ark-DPI vaccine virus failed to replicate in birds and provide adequate protection against pathogenic type Ark-DPI challenge, while eye-drop vaccine application was still efficient at replication and provided protection. To understand the poor Ark-DPI replication in spray applications, we further examined the commercial Ark-DPI vaccines using an electron microscope. We found that vaccine viruses were not mechanically damaged during spray application, rather Ark-DPI vaccine contained many virus particles with less intact spikes compared to the Mass type vaccine. Still, it is not clear why Ark-DPI vaccine virus only fails to replicate in birds when it applied by spray cabinet, but it is possible that the morphological difference of Ark-DPI vaccine viruses may attribute to efficiency of viral replication in birds.

In conclusion, we developed the diagnostic tools that are applicable for detecting and differentiating IBV serotypes. These assays would allow us to quickly identify circulating single or multiple types of IBV in the field and choose the correct vaccine types accordingly. Also the flexibility of the assays and the potential of high-throughput would be beneficial for detecting ever increasing numbers of IBV variants. Through Ark-type vaccine failure studies we gained a better understanding of the dynamics of hatchery vaccination and the behavior of Ark-type vaccine in birds. We now understand that Ark-vaccine is not sufficiently replicating in spray-vaccinated birds but further studies are needed to narrow down what contributes to replication failure of Ark-type vaccine with the spray application. Lack of intact virus particles in Ark-type vaccine could be one of the factors, however there are more explanations needed to figure out the different replication of this vaccine type viruses in embryonated eggs and birds.

Currently, the failure of IBV vaccines, most notably the Arkansas type, to provide adequate protection from challenge in vaccinated birds cannot be linked to any one cause or explanation. New ideas are needed to achieve successful vaccination in the field. One area that

can be evaluated and have a direct impact is assessment of the optimum infectious doses of commercial Ark-DPI vaccines that are currently available in the market and adjusting recommended vaccine doses along with increasing application volumes during hatchery coarse spray vaccination. Parallel to the effort of improving the current vaccine protocol, basic molecular assays need to be performed to elucidate the apparent lack of spike proteins of Ark-DPI vaccine. Basic comparisons of Ark-DPI spike protein density with other viral proteins could be performed to determine the true ratio of spike to other viral proteins for multiple serotypes and highlight the lack of Ark spikes. Evaluating replication patterns of recombinant viruses that express Ark-DPI vaccine virus spikes in other vaccine serotype backbone would help to answer the Ark spike stability question as well as provide a novel subunit vaccine. Ultimately, a solution needs to be found for poultry industry vaccination efficacy that can be put in place sooner rather than later. At the same time, an applied solution will only mediate the problem, whereas obtaining a better working molecular understanding of the virus will allow us to determine the root of the problem and hopefully address it from there.