Novel Infectious Bronchitis Virus Vaccines and Immune Responses

by

Ramon A. Zegpi

A dissertation submitted to the Graduate Faculty of
Auburn University
in partial fulfillment of the
requirements for the Degree of
Doctor of Philosophy

Auburn, Alabama
December 14, 2019

Keywords: Infectious bronchitis virus, vaccine, coronavirus, immune response,
recombinant vaccine, Ark-DPI

Copyright 2019 by Ramon A. Zegpi

Approved by

Haroldo Toro, Chair, Professor, Department of Pathobiology
Vicky L van Santen, Professor, Department of Pathobiology
Kellye Joiner, Associate Professor, Department of Pathobiology
Wallace D. Berry, Associate Professor, Department of Poultry Science

ABSTRACT

Infectious bronchitis virus (IBV) occurs as multiple serotypes and genotypes worldwide. In the United States IBV belonging to the Arkansas (Ark) Delmarva Poultry Industry (DPI) genotype is the most frequently isolated genotype despite extensive vaccination with ArkDPI-derived vaccines. ArkDPI vaccines are heterogeneous, and distinct viral subpopulations become predominant in vaccinated chickens. Vaccine heterogeneity and selection of viral subpopulations after vaccination are likely responsible for the continuous isolation of ArkDPI vaccine-like strains from outbreaks of disease.

We previously demonstrated that adaptation of an embryo-attenuated IBV ArkDPI-derived vaccine to chicken embryo kidney cells (CEK) shifted the virus population towards homogeneity in spike (S) and non-structural protein (NSP) genes. Moreover, the typical Ark vaccine subpopulations emerging in chickens vaccinated with commercial Ark vaccines were not detected in chickens vaccinated with CEK-adapted vaccine virus. Using both conventional and next generation sequencing, we now demonstrate that the changes achieved during CEK adaptation remained after back passages in embryos. We further demonstrate that 1-day-old chickens vaccinated with the CEK-adapted virus are protected against Ark virulent challenge. Moreover, we compared protection conferred by the CEK-adapted virus with the protection conferred by an attenuated commercial ArkDPI-derived vaccine different from which the CEK-adapted virus originated. All vaccinated chicken groups showed a significant reduction of respiratory signs and viral load after Ark virulent challenge compared to unvaccinated-challenged controls. In CEK-adapted virus vaccinated chickens, viral subpopulations different from the challenge virus were detected after challenge in a marginal number (7-8%) of chickens. In contrast, IBV S1 sequences

that differed from the predominant population in the challenge virus were detected after challenge in a large number (77%) of chickens vaccinated with the commercial Ark attenuated vaccine. The CEK-adapted IBV ArkDPI-derived vaccine is a stable and effective vaccine, which drastically reduces the emergence of Ark-like viruses both at vaccination and after challenge.

To further understand the impact of population genetic structure on generation of immune responses and protection, we used comparison of responses to the homogeneous population of the CEK-adapted ArkDPI vaccine and the commercial ArkDPI from which the CEK adapted virus originated as a model. In a first experiment, vaccinated chickens were challenged with an IBV Ark99-type virulent strain (AL4614/98). Despite extensive sequence similarity between the vaccines, the more heterogeneous commercial ArkDPI was more efficient at reducing viral loads in challenged chickens, while respiratory signs and tracheal lesions were reduced similarly by either vaccine. A distinct subpopulation of the Ark challenge virus showing asparagine at S1 position 56 was consistently negatively selected by immune pressure originating from vaccination with either vaccine. Antibody levels and antibody avidity to Ark-type S1 protein were greater in CEK-ArkDPI-vaccinated chickens compared to chickens vaccinated with the more diverse commercial ArkDPI vaccine. Synchronous replication of a homogeneous virus population likely elicits clonal expansion and affinity maturation of a greater number of responding B cells compared to a diverse virus population continuously changing its proportion of phenotypes during replication. The results of a second experiment showed that during initial vaccine virus replication in chickens (24 and 48 hrs post-vaccination), the virus population showing increased diversity (commercial ArkDPI vaccine) achieved higher concentrations of IBV RNA in the trachea compared to the more homogenous CEK-adapted virus. mRNA expression in the trachea of genes associated with immune responses generally showed greater upregulation 48 hrs post-vaccination

in chickens vaccinated with the heterogeneous commercial ArkDPI vaccine compared to the CEK-adapted virus. The greater upregulation of these genes is likely associated with higher virus replication achieved by the heterogeneous commercial vaccine. Thus, while the adaptive antibody response was favored by the more homogenous structure of the CEK-ArkDPI vaccine population (higher antibody levels and antibody avidity), the innate immune response was favored by the more diverse viral population of the commercial ArkDPI. We confirmed previous results that distinct subpopulations in the Ark challenge virus become selected by immune pressure originating from vaccination and concluded that the population structure of IBV vaccines impacts innate immune response, antibody avidity, and protection.

We also explored expression of an immunologically important IBV protein from a vector as a possibly better vaccine alternative to protect chicken flocks against Ark-type virus. A recombinant Newcastle disease virus (NDV) LaSota (LS) expressing secreted trimeric spike (S)-ectodomain (Se) of IBV (rLS/IBV.Se) was developed and evaluated for protection conferred against IBV challenge. The IBV S-ectodomain protein, which is S excluding the transmembrane anchor and short cytoplasmic domain of S2, expressed from recombinant LS corresponds to an Ark-type IBV. In a first experiment, chickens were primed at 1-day of age or primed at 1 day-old and boosted at 14 days-old with 10^4 EID_{50}/bird of rLS/IBV.Se and challenged with a virulent Ark strain. While single vaccination proved completely ineffective at protecting chickens against challenge, priming and boosting reduced clinical signs and tracheal lesions but did not reduce viral load in lachrymal fluids. In experiment 2, the vaccine dose was increased to 10^7 EID_{50}/bird and a different virulent Ark strain was used for challenge. In addition, chickens were singly immunized on either day 1 or day 10 after hatch. NDV antibody levels detected in vaccinated chickens were moderate, with hemagglutination inhibition titers varying between 4 and 5 $\log_2$. Slightly higher

antibody levels to NDV were observed in chickens vaccinated on day 10 versus day 1 but without the difference achieving statistical significance. In contrast, antibody responses measured using recombinant IBV S1 protein-coated ELISA plates were significantly greater in chickens vaccinated on day 10 compared to day 1. The use of a higher rLS/IBV.Se dose proved to enhance the success of single vaccination substantially compared to experiment 1. Signs and tracheal lesions were reduced more effectively in chickens vaccinated at day 10 after hatch. However, as in experiment 1, vaccination did not reduce the viral loads in tear fluids of challenged chickens. Similar results, in which no reduction in viral load in the trachea was apparent following rLS/IBV.S vaccination, have been obtained by others. Further work is needed to understand the immune responses induced by this recombinant virus that seems to provide some protection against the disease but does not reduce viral loads in the upper respiratory tract.

Finally, we explored the possibility that failure of vaccination might be due to the fact that chickens are vaccinated immediately after hatch. Previous work in our laboratory had shown that antibody responses were reduced in chickens vaccinated at 1 day of age compared to chickens vaccinated beyond 1-day of age. In the present work, we examined cross-protection and immune responses elicited by IBV vaccination on day 1 of age or at later time points. Chickens were vaccinated with a Massachusetts (Mass)-type vaccine and heterologous challenge performed with an Ark-type virulent strain. In experiment 1, chickens vaccinated on day 1 or 10 of age were challenged 21 days after vaccination. Analysis of tracheal histopathology and viral load demonstrated enhanced cross-protection when vaccination was postponed beyond day 1 of age. In experiment 2, chickens were vaccinated on day 1 or 14 days of age. A somewhat stronger systemic antibody response to IBV was detected in chickens vaccinated at 14 days of age. In addition, avidity of antibodies to Ark-type S1 protein elicited by vaccination at 14 days of age was greater.

Few differences were noted between chickens vaccinated at 1 or 14 days of age in immune cell populations in the Harderian gland (HG) at the time of sampling 35 days following vaccination. In birds 7 days after challenge both non-vaccinated/challenged groups showed significantly higher (P<0.05) proportions of B cells and $CD8^+$ T cells than age-matched vaccinated/challenged groups or age-matched non-vaccinated/non-challenged control groups. These results indicate infiltration and/or expansion of B cells and $CD8^+$ cells in HGs following challenge of non-vaccinated chickens. A fortuitous finding was that the more immature immune system of 1-day-old chickens was less effective at clearing vaccine virus after vaccination. Collectively, the current results indicate that IBV vaccination at least ten days after hatch induces more effective cross-protection than vaccination on day of hatch. Greater antibody affinity maturation likely contributes to increased cross-protection.

TABLE OF CONTENTS

ABSTRACT 2

TABLE OF CONTENTS 7

LIST OF TABLES 9

LIST OF FIGURES 10

LIST OF ABBREVIATIONS 13

CHAPTER 1. Literature Review 15

1.1. Introduction 15

1.2. History of Infectious Bronchitis Virus 17

1.3. IBV Genome 18

1.4. IBV Replication 20

1.5. IBV Pathogenesis 21

1.6. IBV Immunity 23

1.7. Vaccination against IBV 24

Research Objectives 30

CHAPTER 2. Kidney cell-adapted infectious bronchitis ArkDPI vaccine is stable and protective 31

2.1. Summary 31

2.2. Introduction 32

2.3. Materials and Methods 33

2.4. Results 37

2.5. Discussion 41

CHAPTER 3. Infectious bronchitis virus vaccination at day one of age further limits cross-protection 52

3.1. Summary 52

3.2. Introduction 53

3.3. Materials and Methods 54

3.4. Results 59

3.5. Discussion 62

CHAPTER 4. Infectious bronchitis virus population structure defines immune response and protection 73

4.1. Summary 73

4.2. Introduction 74

4.3. Materials and Methods 76

4.4. Results 82

4.5. Discussion 85

CHAPTER 5. Protection conferred by IBV S-ectodomain expressed from recombinant NDV LaSota 98

5.1. Summary 98

5.2. Introduction 99

5.3. Materials and Methods 101

5.4. Results. 105

5.5. Discussion 107

CHAPTER 6. Conclusions 115

References 116

LIST OF TABLES

Table 2.1. Deduced aa sequences of the S1 glycoprotein recovered from chickens after Ark virulent challenge 45

Table 4.1 Comparison of deduced aa sequences of the S1 glycoprotein recovered from chickens after Ark virulent challenge in two different experiments 89

LIST OF FIGURES

Figure 2.1. IBV RNA levels determined by qRT-PCR in allantoic fluids (AF) during back-passages in embryonated chicken eggs....................46

Figure 2.2 . Differences in deduced aa frequencies in NSP and S proteins of CEK-adapted ArkDPI-derived vaccine after five embryo back-passages compared to previously reported frequencies in commercial vaccine and after one embryo back-passage 47

Figure 2.3. Respiratory signs 5 and 7 days post-challenge with virulent Ark in chickens vaccinated with CEKp7e2 48

Figure 2.4. IBV RNA quantitated by RT-PCR in tears of individual chickens 5 and 7 days post-challenge with virulent Ark in chickens vaccinated with CEKp7e2 49

Figure 2.5. B lymphocytes secreting IBV-specific IgA in the Harderian gland detected by ELISPOT seven days post-challenge in chickens vaccinated with CEKp7e2. 50

Figure 2.6. Respiratory signs and viral load in tears on day 5 after Ark challenge in chickens vaccinated on day 1 of age with CEKp7e2 or ArkDPI-type commercial vaccine. 51

Figure 3.1. Viral load in lacrimal fluids 5 days post-challenge in chickens vaccinated with a Mass-type vaccine and challenged with a virulent Ark-type strain 66

Figure 3.2. Tracheal lesions after virulent Ark challenge in chickens vaccinated with a Mass-type vaccine on Day 1 or 10 of age 67

Figure 3.3. Viral load in lacrimal fluids 5 days post-challenge in chickens vaccinated on Day 1 or 14 post-hatch and challenged 28 days post-vaccination 68

Figure 3.4. IBV antibodies in sera expressed as S/P ratio determined by Mass IBV ELISA and Ark-S1 antibodies determined by recombinant Ark-S1 protein ELISA 35 days after vaccination on Day 1 or 14 post-hatch with a Mass-type vaccine 69

Figure 3.5. Avidity of IBV and Ark-S1 antibodies in sera 35 days after vaccination with a Mass-type vaccine on Days 1 or 14 of age 70

Figure 3.6. Relative frequency of B, $CD4^{+}$, $CD8^{+}$, and $CD8^{+}CD44^{+}$ cells in HGs of chickens 35 days after vaccination (7 days post-challenge for challenged groups) on Days 1 or 14 of age with a Mass-type vaccine 71

Figure 4.1. Protection conferred by CEK-ArkDPI or commercial ArkDPI against homologous challenge with virulent IBV 90

Figure 4.2. Tracheal histopathology and histomorphometry of chickens 91

Figure 4.3. Examples of S1 sequence chromatograms of IBV RNA contained in tears from unvaccinated and vaccinated chickens 5 days after challenge 92

Figure 4.4. Percent tyrosine codon at S1 aa position 56 in IBV RNA in tears of vaccinated and unvaccinated chickens 5 days after challenge with virulent AL/4614/98. Dotted line indicates percent tyrosine (Y) codon in challenge virus stock prior to replication in chickens..................93

Figure 4.5. Ark S1 antibodies and antibody avidity in sera 22 days after vaccination at 14 days of age with the CEK-adapted ArkDPI or the commercial ArkDPI vaccines 94

Figure 4.6. Viral load in tears and tracheas 24 and 48 hrs after vaccination with CEK-ArkDPI or commercial ArkDPI 95

Figure 4.7. mRNA expression of innate immune genes in the trachea of individual chickens vaccinated with the CEK-ArkDPI or the commercial ArkDPI vaccines 96

Figure 4.8. Ribbon model of AL/4614/98 S1 N-terminal domain structure generated by I-TASSER structural homology modeling based on M41 S structure...97

Figure 5.1. Detection of IBV S-ectodomain protein expression from recombinant NDV LaSota by immunofluorescence 111

Figure 5.2. Protection conferred to chickens by prime at 1 day of age and booster at 14 days of age with 10^4 EID_{50}/bird of rLS/IBV.Se against Ark virulent challenge 28 days post-vaccination ...111

Figure 5.3. NDV serum antibodies and incidence of IBV S1 serum antibodies in chickens detected 39 days after single vaccination in chickens with rLS/IBV.Se at 1 or 10 days of age. NDV antibodies by commercial ELISA or byhemagglutination inhibition test.................................112

Figure 5.4. Protection conferred to chickens by single vaccination on day 1 or 10 of age with rLS/IBV.Se against Ark virulent challenge 20 days post-vaccination......................................113

Figure 5.5. Tracheal histomorphometry 5 days post-challenge in chickens singly vaccinated on day 1 or 10 of age with rLS/IBV.Se..114

LIST OF ABBREVIATIONS

3CLpro = 3-chymotrypsin-like protease

aa = amino acid

AAALAC = association for assessment and accreditation of laboratory animal care

ABS = anti-bursal serum

AF = allantoic fluid

ANOVA = analysis of variance

Ark = Arkansas

ArkDPI = Ark Delmarva Poultry Industry

ATS = anti-thymus serum

BSA = bovine serum albumin

CEK = chicken embryonic kidney

DPC = days post-challenge

E = envelope protein

ECE = embryonated chicken egg

EID_{50} = 50% embryo infectious dose

ELISA = enzyme linked immunosorbent assay

GALT = gut associated lymphoid tissue

GC = germinal center

gRNA = genomic RNA

HALT = head associated lymphoid tissue

HEK = human embryonic kidney

HG = Harderian gland

HI = hemagglutination inhibition

IB = infectious bronchitis

IBV = infectious bronchitis virus

IFN = interferon

Ig = immunoglobulin

IL = interleukin

M = membrane protein

MALT = mucosa associated lymphoid tissue

Mass = Massachusetts

MBL = mannose binding lectin

mRNA = messenger RNA

Mx = Myxovirus-resistance

N = nucleocapsid protein

NDV = Newcastle disease virus

NIBV = nephropathogenic IBV

NSP = non-structural protein

OD = optical density

ORF = open reading frame

PBS = phosphate buffered saline

PCR = polymerase chain reaction

PLP = papain-like protease

qRT-PCR = quantitative reverse transcriptase polymerase chain reaction

rLS = recombinant LaSota virus

rLS/IBV.Se = rLS expressing IBV S-ectodomain

rNDV = recombinant NDV

rRNA = ribosomal RNA

RTC = replicase transcriptase complex

RT-PCR = reverse transcriptase PCR

S = spike

S/P = sample to positive ratio

SD = standard deviation

SFC = spot forming cells

snoRNA = small nucleolar RNA

SPF = specific pathogen free

TLR = toll like receptor

TRS = transcriptional regulatory sequences

UTR = untranslated region

CHAPTER 1.

Literature Review

1.1. Introduction

Infectious bronchitis (IB), a highly contagious respiratory disease of chickens, was first described in the United States as a disease of young chickens (121). The etiological agent is IB virus (IBV), a coronavirus from the realm Riboviria, order Nidovirales, suborder Cornidovirineae, family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Gammacoronavirus* and subgenus *Igacovirus* (85). IBV causes relevant economic losses to the poultry industry worldwide.

Live attenuated vaccines are commonly used to control the disease. Experimental evidence shows sizeable cross-protection between different vaccine serotypes (143), but it is generally accepted that vaccines similar to field strains confer better protection. IB control is complicated by the existence of different serotypes that are endemic in all intensive poultry producing regions (24, 145). In the United States, Arkansas (Ark)-type IBV is highly prevalent despite extensive vaccination with Ark Delmarva Poultry Industry (ArkDPI)-derived vaccines (74, 114). Compared to other commercially available IBV vaccines, ArkDPI vaccines show greater genetic diversity, and different viral subpopulations become quickly selected in chickens after vaccination (55, 104, 151). These selected minor viral subpopulations seem to be the source for vaccine-like viruses (76). Viral subpopulations also emerge from challenge virus following homologous challenge in chickens vaccinated with ArkDPI vaccines (64, 144). Thus, ArkDPI-derived vaccines seem to contribute substantially to the emergence of Ark-like variants in the industry.

Previous work in our lab showed that seven passages of an embryo-attenuated IBV ArkDPI-derived vaccine in chicken embryonic kidney (CEK) cells induced genetic and phenotypic

changes in the vaccine. Adaptation to CEK cells made the virus population become more homogeneous. Changes in S protein sequence were not reverted after one back-passage in embryonated chicken eggs (ECE) or after a passage in chickens (63). We aimed at evaluating this more homogeneous vaccine in terms of stability during further back passages in ECE, and protection against homologous challenge. We also determined and compared virus subpopulations emerging after virulent Ark challenge in chickens vaccinated with the CEK-adapted virus or a commercial embryo-attenuated ArkDPI-derived vaccine.

A possible explanation for the occurrence of Ark-like variants after challenge is that the heterogeneous viral subpopulations contained in ArkDPI-derived vaccines affect the quality and quantity of induced immune responses (111, 112). To elucidate the effect of viral population structure on immune response we evaluated the mRNA expression of innate immunity genes in chickens vaccinated with either the CEK-adapted vaccine or the commercial ArkDPI vaccine that originated it.

Current IBV prevention practices involving vaccination on day 1 of age may also contribute to suboptimal immune responses. Numerous studies since the early 1970s have shown that the immune system of 1-day-old chickens is not fully developed (3, 21, 100, 113, 118, 123, 156, 157). More recently, van Ginkel *et al.* (148) showed that the concentration of IBV-specific class G immunoglobulin (IgG) in plasma and lachrymal fluid increases significantly if vaccination is performed on day 14 instead of day 1 of age. Saiada *et al.* (119) showed that vaccination on day 1 of age elicits lower antibody responses compared with vaccination at later time points. We evaluated the effect of age of vaccination on IBV cross-protection. Chickens vaccinated with a Massachusetts-type vaccine at increasing age were challenged with an Ark-type virulent strain.

Vectored vaccines, which allow expression of immunodominant epitopes, seem to be excellent candidates to replace live-attenuated vaccines. Recombinant Newcastle disease virus (rNDV) encoding IBV's S1, S2 and S has been developed and tested to protect chickens against virulent IBV challenge. rNDV expressing the S2 proteins provided protection against virulent IBV challenge (147). Protection provided by rNDV expressing the whole S protein of IBV M41 strain provided better protection than rNDVs expressing S1 or S2 alone (128). In previous work, chickens were primed and boosted with recombinant IBV S-ectodomain protein emulsified in Montanide ® ISA 71 VG adjuvant (45). Chickens vaccinated with the S-ectodomain protein, i.e. S1 with the exposed N-terminal domain and S2 without the cytoplasmic tail, showed specific antibody responses and reduced viral loads and tracheal lesions upon challenge. Our current work evaluates the protective efficacy of rNDV expressing the S-ectodomain against virulent IBV challenge.

1.2. History of Infectious Bronchitis Virus

Schalk and Hawn (121) first reported IB as a novel respiratory disease that caused high mortality in young chickens. It was later differentiated from laryngotracheitis and coryza using cross-immunity tests by Beach and Schalm (12). In 1937, Beaudette and Hudson (14) propagated IBV in chicken embryos and established that virus became more lethal for embryos after continued passaging. The first serologic method established for IBV diagnosis was developed by van Roeckel in 1942 (reviewed in (50)). The serum neutralization test for IBV in embryonated chicken eggs uses the Beaudette strain, which is lethal for chicken embryos. Non-lethal IBV strains cause stunting and curling of embryos in IBV infected fertile eggs (50); those lesions are considered pathognomonic. The possibility of immunizing flocks against IBV was conceived from field observations indicating that chickens infected at early ages (8 to 16 weeks) exhibited mild

symptoms and were protected against the disease later (50). The first demonstration of antigenic variation among IBVs was reported by Jungherr *et al.* (80). They observed that two isolates obtained from different locations in different years produced a similar disease but neither cross-protected nor was cross-neutralized. This finding highlighted that a vaccine serotype had to be similar to the serotype of the field strain in order to induce effective protection. This finding is of importance today because several serotypes coexist in poultry producing regions, which complicates vaccination programs. Control of IBV is further complicated by high-density chicken populations, continuous growth of the poultry industry, IBV high mutation rates, and heterogeneous live vaccines used to prevent outbreaks of disease.

1.3. IBV Genome

The coronavirus genome is the largest among all RNA viruses, ranging from 26 to 32 kilobases. The single-stranded, positive-sense non-segmented RNA acts as mRNA, template for RNA replication, and substrate for packaging into new viral particles (102). The genome comprises a basic set of genes with a conserved order 5'-replicase-S-E-M-N-3'. In the specific case of IBV, the RNA genome is approximately 27.6 kilobases long. The gene organization is 5' untranslated region 5'-(UTR)-leader-1a/1ab-S-3a/b-E-M-4b/c-5a/b-N-6b-3' UTR (6, 19, 26, 137). Transcriptional regulatory sequences (TRS) are present before each protein-coding gene (6) and regulate gene expression during infection.

The IBV genome encodes four structural proteins: spike (S), small envelope protein (E), membrane glycoprotein (M) and nucleocapsid (N). Among all structural proteins, the most studied is the S glycoprotein. It is a class I viral fusion protein (18). This trimeric protein located on the virion's surface is post-translationally divided into subunits S1 and S2 (93, 135). The cleavage site

corresponds to the amino acid motifs RRFRR, RRSRR or RRHRR (133). The N-terminal S1 subunit is responsible for attachment of the virion to the host cell (25, 83, 102). The S1 subunit induces virus-neutralizing antibodies in the host (29, 83). The carboxyl-terminal subunit S2 anchors S1 to the virion and orchestrates membrane fusion with the host cell (26). The E glycoprotein is abundant in the Golgi complex and seems to be necessary for virus assembly (35). The M glycoprotein is the predominant constituent of the viral envelope (53). The N protein is found in the ribonucleoprotein core (102). It plays a crucial role in viral replication and assembly (122).

The non-structural genes 1a and 1b are located in the 5' end of the genome and cover two-thirds of the total genome. In IBV, these genes encode 15 proteins, non-structural protein (NSP) 2 to 16. A -1 frameshift mechanism is required for the two large overlapping open reading frames (ORF) 1a and 1b to be translated together into two large polypeptides that are cleaved by viral proteases (20, 52, 102). The frameshift depends on a conserved pseudoknot RNA structure that separates 1a and 1b (110). NSP3 encode two papain-like (PLP) proteases, but only one is active (173). This protease is involved in autocatalytic release of NSP3 PLP. The NSP5 gene encodes the 3-chymotrypsin-like protease (3CLpro), also called main protease (Mpro) (172). NSP4 and NSP6 participate in 3CLpro autocatalytic release (138). NSP9 appears to have single stranded RNA binding activity (31) which may protect viral RNA from degradation. NSP12 is the RNA-dependent RNA polymerase (RdRp) (99) that forms a complex with NSP8 (136). NSP13 has helicase activity, necessary for viral replication (51). NSP14 is an exonuclease associated with proofreading activity (131) and forms a complex with NSP10 (99). Nsp15 is predicted to be a poly (U)-specific endoribonuclease associated with production of mature small nucleolar RNAs

(snoRNAs), which may be utilized in different rRNA processing tasks. Nsp16 is predicted to assist in rRNA methylation (132). There is no clear function for NSP2, NSP7 or NSP11.

Genes encoding accessory proteins are distributed in-between the structural protein genes at the 3' end of the genome. IBV possess seven predicted accessory protein genes: 3a, 3b, 4b, 4c, 5a, 5b and 6b (26, 27, 68). The function of accessory proteins is not fully elucidated, but 5b and 3b have been implicated in host immune response evasion by IBV (86, 87). Protein 3b inhibits activation and translocation of STAT1 and protein 5b function similarly to NSP1 of *Alpha*- and *Betacoronaviruses*.

1.4. IBV Replication

As in other members of the family *Coronaviridae*, replication of IBV occurs in the cytoplasm of the host cell and the presence of membranous compartments is necessary (90, 102). For the infection to occur, virions must attach to host cell receptors through the S protein. Attachment induces a conformational change in the spike that results in fusion of the viral envelope and the host cell membrane. Subsequently, the viral nucleocapsid containing the viral genome is released into the cell. Polyproteins 1a and 1ab are transcribed first from the original genome. These polyproteins are cleaved into 15 NSP (NSP2 to NSP16) by PLP, encoded by the nsp3 gene (172, 173), and 3CLpro, encoded by the nsp5 gene (172). The RdRp along with other viral proteins form the replication-transcription complex (RTC) (99). The RTC transcribes nested, negative sense RNAs, including a full-length copy of the genome. Negative sense RNAs are used as templates to synthetize positive sense genomic RNA (gRNA) and positive sense sub-genomic RNA (sgRNA), referred to as sgRNA 1 to 7 (102).

All structural proteins are produced from sgRNAs (11, 120). Each sgRNA consists of a leader RNA sequence 70 to 100 bases long, identical to the 5’ end of the genome, joined to a body RNA which is identical to a segment of the 3’ end of the genome. Like the original genome, sgRNAs have 5’ caps and 3’ polyadenylate tails (102). Transcriptional regulatory sequences (TRSs) are short motifs where the fusion between the leader and the body sequences occur. In IBV the sequence of the TRS is CU(U/G)AACAA (A/T) (6). After the RTC reaches a TRS, the RdRp will follow one of two paths, continue with transcription or relocate to the leader TRS motif sequence at the 5’ end of the genome through base-pairing interactions between the anti-TRS of the negative sense strand and the leader TRS (117). This discontinuous transcription results in smaller, sub-genomic negative sense sequence transcripts. In the cytoplasm, gRNA and N protein combine to form the helical nucleocapsid (134). Assembly of structural proteins S, M and E occurs in the rough endoplasmic reticulum and the Golgi apparatus (90). Virions are released when vesicles carrying viral particles fuse with the cytoplasmic membrane (134). The replication mechanism of Coronaviruses makes them prone to high rates of recombination, which plays an important role in IBV evolution (137). IBV genetic variability is also caused by the high mutation rate inherent to RNA viruses (137).

1.5. IBV Pathogenesis

IBV clinical disease has an incubation period of 24 to 48 hours. Morbidity is usually high, reaching over 90%, because IBV rapidly spreads in a flock by aerosol and mechanical means. Mortality depends on the virulence of the infecting IBV, chicken age, host immune status, and secondary bacterial infections, among other factors. Mortality varies from minimal in 6 week-old chickens and older to up to 25% in chickens under 6 weeks of age or as high as 1% weekly in cases involving nephritis-nephrosis (73).

The respiratory tract is the primary site of IBV replication, although the virus has tropism for several tissues (4, 39, 70). Clinical disease has been extensively described for the upper respiratory tract, kidneys and oviduct. IBV replication in the trachea causes a highly contagious respiratory disease characterized by loss of cilia and desquamation of tracheal epithelium (56, 80, 109). As a result, non-specific signs such as gasping, coughing, sneezing, tracheal rales, facial edema, and nasal discharge occur (23, 116). Damage to the respiratory epithelium predisposes the chickens to secondary infections, e.g. E. coli, causing airsacculitis and colibacillosis (103).

Nephropathogenic strains produce less severe respiratory signs (174) but cause nephritis with mortality rates as high as 90% (72). IBV replication in kidneys of chickens induces dilation of the distal convoluted tubules, collecting tubes, and collecting system of both cortex and medulla, followed by inflammatory changes such as interstitial mononuclear cell infiltration and tubular damage (129). Nephropathogenic IBV outbreaks can be confounded and/or intensified by poor management such as high dietary calcium (65).

In layer and breeder chickens, IBV infection of the oviduct decreases egg quality and production (34, 36, 126). IBV replication in the oviduct causes lasting harm in immature females, producing reduced egg production and birds failing to produce eggs. Eggshells may lose pigmentation and the albumen may show watery viscosity. Egg production may be permanently depressed after IBV infection in highly susceptible flocks.

Outbreaks of proventriculitis associated with IBV have been reported frequently in China with varying mortality rates (163). IBV also replicates in other tissues, but without significant consequences for poultry production. Infection of testicles (17, 54) and enteric tissues occurs (4, 33, 98, 115, 163), usually without clinical manifestation.

1.6. IBV Immunity

During the early phase of a primary IBV infection, innate immune and Th1-based adaptive responses are induced to clear the virus from the trachea (155). Toll-like receptor (TLR) 3, TLR7, and TLR2/6 recognize IBV during the innate immune response in the trachea (66, 155). Mannose-binding lectin (MBL) binds to IBV early during infection (88), which likely involves MBL in opsonization of free viral particles, complement activation, or inhibition of the viral entry to host cells (66, 81, 82, 171). IBV recognition by the innate immune system leads to a plethora of pathways that cause types I and II interferon activation, triggering the antiviral state of neighboring cells and the entry of T cells to the local tissue (84).

IBV infection induces antibody production in chickens. Humoral immunity depends on antibodies that can be detected in serum and tears between one and two weeks post-infection. The magnitude of antibody response depends on age (37), maternally derived antibodies (107), and immunosuppression (145) among other factors, as reviewed in (9, 44, 73). Immunoglobulin (Ig) A, IgG and IgM can be detected in serum (60, 96, 106). IgA and IgG are commonly detected in lachrymal fluid and tracheal washes (8, 61). Most of the IgA in tears originates from the Harderian glands (8, 41). IgG presence in tears results from passive transport from the serum (41, 140). IgM is present for a short period after inoculation and it is an indicator of a recent vaccination or infection (42). It is generally accepted that serotype-specific virus neutralizing antibodies are only induced by the amino-terminal S1 subunit of the spike glycoprotein (28), although protection conferred by vaccination with the S2 subunit of the S protein vectored in a recombinant NDV virus has been reported (46, 147).

In cell mediated immune responses, cytotoxic T lymphocytes attain a better initial elimination of virus from kidneys and lungs during acute IBV infection than the humoral IgM and IgG response (124). Transfer of immune T cells to chicks prior to IBV infection showed that IBV-primed cytotoxic T lymphocytes with αβ T cell receptors protected chicks from acute respiratory IBV infection (125).

1.7. Vaccination against IBV

Live and inactivated virus vaccines are used in the poultry industry for IBV immunization. Live vaccines are commonly used at day 1 of age. IBV strains used for live vaccines are generally attenuated by passages in embryonating chicken eggs (16). Homologous protection against IBV in chickens has been documented, but the extent of cross-protection against other IBV strains (heterologous protection) varies. Age of vaccination has been described as an important factor of vaccination outcome (43), although vaccination practices in the field are determined by technical reasons more than scientific. Live-attenuated vaccines may produce unwanted new vaccine-like strains. In contrast, vectored vaccines are a promising option in terms of solving vaccine's stability and specificity problems.

Cross-protection. Several studies have reported cross-protective immunity induced by vaccination after challenge with heterologous IBV strains. Winterfield *et al.* (161) showed that a Massachusetts type vaccine (H strain) induced different degrees of protection against challenge with 8 different IBV serotypes. A similar experiment implemented by Hofstad (69) evaluated cross protection between 7 different IBV serotypes by assessing tracheal virus shedding reduction. The outcome revealed variable cross-protection between serotypes. Darbyshire (38) evaluated cross protection between the H120 vaccine strain and seven heterologous challenge strains of IBV by

determining ciliary activity of tracheal explants. Vaccinated chickens were protected against strains of the Massachusetts M-41, Connecticut 46, Holte, and Iowa 609 types and there was incomplete protection against Iowa 97 and Gray strain. Gelb *et al.* (62) described cross-protection among five field IBV isolates obtained from broilers and eight different anti-IBV sera. Endo-Muñoz and Faragher (47) showed that vaccination with four different IB vaccine viruses induced different degrees of reduction of ciliostasis caused by Australian isolates N1/62 and N9/74, having best results with native vaccine strains. Klieve and Cumming (89) determined that four out of six Australian IBV isolates significantly reduced tracheal ciliostasis after challenge with a different nephropathogenic IBV (NIBV) strain. Lambrechts *et al.* (92) showed that vaccination with NIBV induced protection against homologous and heterologous NIBV challenge, while H52, H120 and D274 vaccination shortened the period of replication of the challenge virus in the trachea. In another cross-protection study, Lim *et al.* (94) evaluated protection induced by commercial vaccines containing H120 or K2 strains against new isolates that emerged from recombination events between Korean (such as K2) and Chinese IBV strains. The H120 strain did not provide protection against variants, whereas the K2 strain, closer to the challenge strains, did. In previous work in our lab, Toro *et al.* (143) evaluated protection against IBV in chickens primed or primed and boosted with a Mass-type attenuated vaccine when challenged with IBV Arkansas (Ark) or GA13-type virulent strains. Analysis of clinical signs, viral load, and histopathology revealed significant cross-protection among these antigenically distant IBV strains. Booster vaccination with Mass after priming with Mass improved protection against GA13 and Ark compared to only prime vaccination with Mass.

Age of vaccination. An important factor to consider when evaluating vaccination efficacy is age of vaccination (43). Albini and Wick (3) determined the percentage of lymphoid cells reacting

with anti-bursal serum (ABS) or anti-thymus serum (ATS) in lymphoid organs obtained from chickens of different ages. There was a significantly higher number of ATS reacting cells in the Harderian gland of 9-week-old chickens compared to younger birds. The proportion of ABS-reacting cells remained similar as chicken aged. The development of B and T cell germinal centers (GC) in chickens has also been described as age dependent. Jeurissen *et al.* (77) observed an increase of B and T cell GC in mucosa associated lymphoid tissue (MALT) of SPF chickens as early as 2 weeks of age for the bronchus and associated lymphoid tissue, Meckel's diverticulum, cecal tonsils and Peyer's patches. The Harderian gland presented an increase of GCs from 6 weeks on, coupled with the appearance of plasma cells. Presence of B cells was detected in all MALTs from 5 days of age and onwards with little variation. Similar results were obtained by Albini *et al.* (3). The results obtained by Lowenthal *et al.* (97) also indicate an age dependent T cell maturation process. Their results show that 1-day-old chicken T cells are functionally immature as evaluated by their inability to proliferate or produce cytokines following immune stimulation compared to adult bird T cells (6 weeks old). In addition, they showed that spleen cells of 1-day-old chickens produced a soluble inhibitor that stopped the proliferation of stimulated adult chicken T cells. The inhibition activity decreased by the second day post-hatch and corresponded with enhanced T cell stimulation. Results of Lowenthal *et al.* (97) suggest a period of transient T cell unresponsiveness to immune stimulation in 1 day old chickens that could be orchestrated by a functional immaturity of T cells and soluble inhibitor(s). Bar-Shira *et al.* (10) assessed the establishment of immune competence in chicken gut associated lymphoid tissue (GALT). Consistent with Lowenthal *et al.* (97), Bar-Shira *et al.* propose that B and T lymphocytes are functionally immature in the GALT immediately after hatch. Maturation of these cells occurs during the first 2 weeks of life as determined by expression of IL-2 and IFNγ mRNA. Likewise, Jeurissen *et al.* (77) described an

increase in lymphocyte populations during the second week of age (77). Maslak and Reynolds (100) studied changes occurring in B cells and T lymphocyte subsets of the head-associated lymphoid tissues (HALT) in chickens ranging from 1 to 8 weeks of age (100). Their results are consistent with those of Albini *et al.* (3) and Jeurissen *et al.* (77), showing that T lymphocytes increased as chickens aged, but the B cell subset showed no significant change in the Harderian gland. Also, Maslak and Reynolds (100) described GCs to be more evident at 4 weeks of age compared to 1-week-of-age chickens, similar to Jeurissen *et al.* (77). Mast and Goddeeris (101) studied at what age broilers develop a humoral response to the thymus dependent antigen bovine serum albumin (BSA). Chicks immunized at 12 days of age showed strong IgM and IgG responses, chickens immunized on day 7 of age had just detectable responses, and broilers immunized *in ovo* or at day 1 of age had no detectable IgM or IgG responses against BSA. Moreover, chickens immunized at day 1 had a lower production of IgG measured during 28 days compared to chickens immunized at day 7 or 14. In addition, 1-day-old immunized chickens did not show increased IgG production after a booster immunization contrary to broilers immunized at day 7 or 14 of age. Their results indicate a poor humoral response after vaccination at day 1 compared with vaccination at 1 or 2 weeks of age. All these findings provide evidence that the immune function of the late embryonic and neonatal chicken is not entirely developed.

Experiments evaluating immune resistance against pathogenic organisms also strongly suggest age as an important factor in chickens. Wells *et al.* (157) described age-dependent activity of chicken heterophils against *Salmonella enteritidis*, a two-fold increase in phagocytic index was shown at day 7 of age compared to 1 or 4 day-old chickens. Antigen-specific T cell response against enteric salmonellosis was also found to be associated with age by Beal *et al.* (13). Birds infected at 40 days of age cleared salmonella infection effectively while chickens infected at 10

days of age were highly susceptible to infection. Furthermore, Michailidis *et al.* (105) used quantitative real-time PCR analysis to measure the expression of TLR mRNAs after *S. enterica* inoculation. TLR5 was significantly increased in the ovaries of older birds (104 weeks old) compared to sexually mature birds (28 weeks old). Crinion and Hofstad (37) evaluated the pathogenicity of four serotypes of IBV for the oviduct of young chickens and described age-associated resistance against IBV. They found that histologic lesions of epithelial cells lining the oviduct lumen were less frequent as the age of inoculated chickens increased. The same tendency was observed in the presence of IBV determined by immunofluorescence staining of the oviduct. Similarly, Albassam *et al.* (2) showed that young birds were much more susceptible to nephropathogenic effects (tubular damage, interstitial inflammatory cell infiltration and edema) of a virulent IBV challenge when compared to older birds. Previous work by van Ginkel *et al.* (148) confirmed that vaccination at a later age improved the protection conferred by a live attenuated ArkDPI IBV vaccine strain against challenge with an Ark serotype IBV field isolate (AL/4614/98) under experimental conditions. A significant increase of IBV-specific IgG and IgA titers was detected in chickens vaccinated at days 7 or 14 of age compared to chickens vaccinated at day 1 of age. Their result was consistent with results of others cited above (10, 77, 101), which indicate that development of the chicken immune system requires at least seven days after hatch for adequate immune responses.

Recombinant virus vaccines. Viral-vectored vaccines elicit humoral and cellular immune responses. Significant advantages such as efficient antigen presentation, high stability and flexibility in protein or epitope selection support the use of these vaccines as candidates to replace live-attenuated vaccines. Additionally, they can be mass produced, and allow the production of multivalent vaccines.

Vaccination with subunit vaccines, such as S1 and N proteins co-expressed by a recombinant baculovirus, or viral vectored vaccines, for instance adenovirus-based vaccines encoding the S1 protein, protected chickens against challenge (78, 165, 168, 169). Newcastle disease virus (NDV) has been used as a vaccine vector expressing IBV proteins S1, S2 and S by different authors (1, 46, 128, 147). Recombinant NDV vaccine (rNDV) expressing the S2 protein provided variable protection against virulent IBV challenge (46, 128, 147). Protection provided by rNDV expressing the whole S protein of IBV M41 strain provided better protection than rNDVs expressing S1 or S2 alone (128).

Research Objectives

1. Evaluate stability of kidney cell-adapted infectious bronchitis ArkDPI vaccine and determine vaccine effectiveness.
2. Determine cross-protection to infectious bronchitis viruses upon vaccination postponed beyond day one of age.
3. Determine how the population structure of infectious bronchitis virus defines immune responses and protection.
4. Evaluate protection conferred by recombinant Newcastle disease virus expressing infectious bronchitis spike-ectodomain protein.

CHAPTER 2.

Kidney cell-adapted infectious bronchitis ArkDPI vaccine is stable and protective

Avian Diseases 61:221-228. 2017

Zegpi, R. A., C. Breedlove, V. L. van Santen, C. R. Rasmussen-Ivey, and H. Toro.

2.1. Summary

We previously demonstrated that adaptation of an embryo-attenuated infectious bronchitis virus (IBV) Arkansas (Ark) Delmarva Poultry Industry (DPI)-derived vaccine to chicken embryo kidney (CEK) cells (CEKp7) shifted the virus population towards homogeneity in spike (S) and nonstructural protein genes. Moreover, the typical Ark vaccine subpopulations emerging in chickens vaccinated with commercial Ark vaccines were not detected in chickens vaccinated with CEKp7, indicating that kidney-cell adaptation drastically increased the stability of the vaccine virus population in chickens. In the current study both conventional and next-generation sequencing results show that the changes achieved during CEK adaptation remained after five back passages in embryonated chicken egg (ECE). In a first protection study 1-day-old chickens were vaccinated with $10^{4.0}$ or $10^{5.0}$ 50% embryo infectious doses (EID_{50})/chicken of the second ECE back passage of CEKp7 (CEKp7e2) and demonstrated protection against Ark virulent ($10^{6.0}$ EID_{50}) challenge. In a second protection trial, protection by CEKp7e2 was compared with protection conferred by an attenuated commercial ArkDPI-derived vaccine different from that which the CEK-adapted virus originated. All vaccinated chicken groups showed a significant reduction of respiratory signs and viral load after Ark virulent challenge compared to unvaccinated-challenged controls. In CEKp7e2 vaccinated chickens, viral subpopulations different from the challenge virus were detected after challenge in a marginal number (7%-8%) of chickens. In contrast, IBV S1

sequences that differed from the predominant population in the challenge virus were detected after challenge in a large number (77%) of chickens vaccinated with the commercial Ark attenuated vaccine. The CEK-adapted IBV ArkDPI-derived vaccine is a stable and effective vaccine, which drastically reduces the emergence of Ark-like viruses both at vaccination and after challenge.

2.2. Introduction

Arkansas (Ark)-type infectious bronchitis virus (IBV) is highly prevalent despite extensive vaccination with embryo-attenuated Ark Delmarva Poultry Industry (ArkDPI)-derived vaccines (74, 114, 145). Vaccine virus subpopulations, which quickly become predominant in chickens after vaccination (55, 104, 151) likely provide a source for the emergence of novel vaccine-like viruses commonly isolated from broiler respiratory disease. In addition to subpopulations originating from vaccine virus, accumulating evidence shows that subpopulations emerge from wild Ark challenge virus in chickens that had been vaccinated with attenuated ArkDPI vaccines (64, 111). Thus, current ArkDPI-derived vaccines contribute to emergence of novel Ark-like variants in the industry. A sound explanation for this phenomenon is that the varying proportions of viral subpopulations contained in attenuated ArkDPI-derived vaccines influence their ability to replicate as well as the subsequent qualitative and quantitative nature of induced immune responses (111, 112).

We previously investigated genetic and phenotypic changes that were associated with the adaptation of an embryo-attenuated IBV ArkDPI-derived vaccine virus to chicken embryonic kidney (CEK) cells. The virus population shifted toward homogeneity in spike (S) and nonstructural (NSP) genes during passages in CEK cells. Based on S gene sequencing, the changes of the predominant Ark population after CEK adaptation were not reverted after one back passage

in embryonated chicken eggs (ECE) or after a passage in chickens (63). Because of the advantages of this more homogeneous CEK-adapted ArkDPI virus, this study was aimed at evaluating its stability during further back passages in ECE, and its ability to confer protection against homologous challenge. Furthermore, we investigated and compared virus subpopulations emerging after virulent Ark challenge in chickens that were vaccinated with the CEK-adapted virus to those emerging in chickens vaccinated with a commercial embryo-attenuated ArkDPI-derived vaccine.

2.3. Materials and Methods

Chickens. White leghorn chickens were hatched from specific-pathogen-free (SPF) embryonated eggs (Wayward Acres, Pickens, SC) and maintained in Horsfall-type isolators in Biosafety Level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

Viruses. The previously described CEK-adapted ArkDPI vaccine virus (CEKp7), which had been back passaged once in SPF ECE (63) was subjected to four further back passages in SPF ECE. The second passage was used at two dose levels to assess protection conferred by vaccination (see experimental designs below). In a second animal trial a commercial embryo-attenuated ArkDPI-type vaccine, different from that from which the CEK-adapted virus originated, was used for comparison. The IBV Ark-type virulent strain (GenBank Accession No. JN861120) previously described (54) was used for challenge purposes. Viruses were titered in ECE as generally accepted (57, 152) including slight modifications previously described (64). In brief, in addition to

macroscopic embryo changes, the embryo weight and IBV RNA detection by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (22) in embryo allantoic fluids (AF) were used to determine the virus titers. Thus, the titration method employed is more sensitive than the generally accepted method. Vaccination and challenge in birds were performed with a total volume of 100 µl (25 µl in each nostril and each eye) of appropriately diluted virus stock.

Stability of CEKp7 during back passages in ECE. The CEKp7, previously passaged once in ECE (CEKp7e1), was further back passaged in SPF ECE to complete five back passages. Passages were performed by inoculating 100 µl, containing AF from the previous ECE passage diluted 1:10 in tryptose broth, into each of 10 SPF ECE. Allantoic fluids were harvested from each individual ECE 3 days after inoculation, clarified by centrifugation, and a portion (1 ml from each egg) pooled for the subsequent egg back passage. Another portion of the AF from five individual ECEs was stored at 80 C until RNA extraction for IBV genome quantitation and spike gene sequencing. Relative IBV RNA concentration in RNA extracted from AF from each egg following each ECE passage was determined by qRT-PCR (22). Conventional sequencing of the S gene was performed after each passage, and IBV whole-genome next-generation sequencing was performed after the fifth passage (described below).

Protection conferred by increasing doses of CEK-adapted IBV ArkDPI-derived vaccine administered at 1 day of age. Four treatment groups were established (*n* = 12-16 per group). Chickens in Groups 1 and 2 were vaccinated with 10^4 EID_{50}/bird and 10^5 EID_{50}/bird, respectively, of the second embryo back passage of CEKp7 (CEKp7e2) at 1 day of age. Chickens in Groups 3 and 4 served as nonvaccinated/challenged and nonvaccinated/nonchallenged controls, respectively. All birds in Groups 1-3 were challenged at 20 days of age with 10^6 EID_{50}/bird of the

virulent IBV Ark. Protection against challenge was evaluated both at 5 and 7 days after challenge by assessment of respiratory clinical signs and viral load in tears. Respiratory rales (nasal and/or tracheal) were evaluated blindly (without knowledge of group) by close listening to each bird and scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe), as described (145). Clinical scoring data were analyzed by Kruskal-Wallis followed by Dunn's post-test. Relative viral loads in lachrymal fluids were determined by relative viral RNA levels measured by qRT-PCR (22). Viral RNA data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey multiple comparisons post-test. Differences were considered significant with $P<0.05$.

Finally, B lymphocytes in the Harderian gland secreting IBV-specific IgA were evaluated by enzyme-linked immuno-spot-forming (ELISPOT) assay as previously described (149) on Day 7 after challenge. However, instead of evaluating cell suspensions from the Harderian glands of individual birds, assays for each group were conducted in four pools of samples each from three chickens; i.e., the resulting values for each group represented a total of 12 chickens, but because of the low number of observations per group (n = 4) no statistical analysis was performed.

Protection and virus subpopulations emerging from virulent Ark challenge strain in chickens vaccinated with CEKp7e2 versus chickens vaccinated with a commercial embryo-attenuated ArkDPI-derived vaccine were evaluated. Five treatment groups were established (n = *15*/group for Groups 1-4; n = 10 for Group 5). Chickens in Group 1 were vaccinated with $10^{4.0}$ EID_{50}/bird of a commercial ArkDPI-derived vaccine at 1 day of age. Chickens in Groups 2 and 3 were vaccinated with $10^{4.0}$ EID_{50}/bird and $10^{5.0}$ EID_{50}/bird of CEKp7e2, respectively, at 1 day of age. Chickens in Group 4 served as non-vaccinated/challenged controls. All birds in Groups 1-4 were challenged at 20 days of age with $10^{6.0}$ EID_{50}/bird of the virulent IBV Ark. Group 5 served

as the non-vaccinated/non-challenged negative control group. Protection against challenge was evaluated by assessment of clinical signs and viral load in tears on Day 5 after challenge, as described above. A portion of RNA extracted from tear fluid of each individual challenged chicken was used for partial S1 gene sequencing.

IBV RNA extraction. IBV RNA to be used for qRT-PCR and S gene sequencing was extracted from AF of each ECE of each CEKp7 back passage, and from tear samples collected from individual chickens in the second protection trial using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. RNA to be used for next-generation sequencing was extracted from AF with the use of TRI Reagent LS RNA Isolation Reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol and omitting the isopropanol precipitation step. RNA was further purified with the use of the Qiagen RNeasy mini kit, following the manufacturer's RNA cleanup protocol.

Sequencing of S genes. RT-PCR was carried out with the use of the Qiagen one-step RT-PCR kit. Primers NEWS1OLIGO5' and S1OLIGO3' (91) were used to amplify the S1 gene and primers S5F (151) and S3' (151) to amplify the S2 gene of IBV from allantoic fluids following egg passages. Primers S17F and S18R (55) were used to amplify a portion of the IBV S1 gene (66 nt 5' to start of translation through nt 728) from tear samples. RT-PCR products were visualized by gel green stain (Phoenix Research, Candler, NC) after agarose gel electrophoresis. Amplified cDNA was submitted to the Massachusetts General Hospital DNA core facility for PCR cleanup and sequencing with S17F, S1R, S2F (151). , and S1OLIGO30 primers for cDNA amplified with primers NEWS1OLIGO50 and S1OLIGO30; S5F, S11F, S7F, and S20R (151) for cDNA amplified with primers S5F and S30; and S17F and S1R for cDNA amplified with S17F and S18R

primers. Sequences of the S gene from embryo back passaged CEKp7 were aligned to the CEKp7 S gene sequence with the use of MacVector software (MacVector Inc., Cary, NC) for comparison. S1 gene sequences of IBV in tears of challenged chickens were aligned to the S1 sequence of the challenge strain for comparison. All sequence chromatograms were examined to identify positions containing more than one peak indicating the presence of a mixed IBV population.

Sequence analyses of CEKp7 after five back passages in ECE by deep sequencing. RNA purified from allantoic fluid that contained CEKp7 after 5 ECE back passages was submitted for next-generation Illumina Sequencing at the Genomic Services Lab at Hudson Alpha Institute for Biotechnology (Huntsville, AL) (50 bp paired-end reads; 15 million reads). The resultant paired-end sequencing data were trimmed with the use of CLC Genomics Workbench Software, with a trim setting (0.01) used to achieve high-quality sequences with low error probability. Trimmed sequences were then used to create a reference assembly with the use of the ArkDPI passage 101 genome (5) (GenBank Accession No. EU418975) as the reference genome with the default setting (0.80) used for sequence match. Single-nucleotide polymorphism detection of nucleotides at .0.1% frequency was then performed on the reference assembly and analyzed with the use of CLC Genomics Workbench. An average of 7508 reads were evaluated at each genome position. Frequencies of nucleotides at each genome position were compared with those previously obtained from the IBV vaccine virus stock and from the CEKp7 after 1 ECE back passage (63).

2.4. Results

Stability of CEK-adapted vaccine virus during back passages in ECE. Viral loads determined in AF during CEKp7 back passages in ECE are shown in Figure 2.1. As seen in this figure, viral loads remained similar during passaging. From a basic perspective, this result is consistent with

lack of adaptation of the virus to the egg environment; i.e., positive selection would have resulted in higher viral concentrations with successive egg passages, and negative selection would have ended in lower titers. From an applied perspective, this result is also relevant, as consistent titers can be expected when producing commercial vaccine batches.

Conventional sequencing of the IBV S genes in the AF of each of five ECE following all back passages showed that all three nucleotide changes observed in the S gene after CEK adaptation were maintained during back passages in embryonated eggs; i.e., reversion was not observed. However, heterogeneity was apparent at one of these positions and three other positions in the S gene and was confirmed by next-generation sequencing (see below).

Figure 2.2 shows differences in deduced amino acid (aa) frequencies in NSP and S proteins of CEK-adapted ArkDPI-derived vaccine after one and five ECE back passages compared to the original vaccine at positions where frequencies differed among the three viral populations, as determined by next generation sequencing. At all seven genome positions where the major aa encoded had changed during adaptation to CEK cells (indicated by italics in the figure), these changes were maintained after five ECE back passages. Furthermore, at all S1 and NSP positions where homogeneity had increased during adaptation to CEK cells the increased homogeneity was maintained, or the degree of homogeneity further increased. For example, at S1 aa position 213, commercial ArkDPI attenuated vaccines show a predominant population containing serine and a subpopulation displaying alanine. After CEK adaptation the displayed frequency of serine is 100% at this position (63) , and this frequency was maintained after five back passages in ECE. In contrast, increased heterogeneity was observed at S1 aa positions 119, 222, and 475 and at a single aa position in NSP2, positions at which changes had not been detected during CEK adaptation.

Only one of the positions where the major aa encoded had changed during CEK adaptation increased in heterogeneity during embryo back passages of the CEK-adapted virus: S aa 889, in the S2 subunit. This aa changed from 100% serine in the original vaccine to 96% phenylalanine and 4% serine after CEK cell adaptation, then increased in heterogeneity to 80% phenylalanine and 20% serine after embryo back passages.

Protection of chickens vaccinated with CEKp7e2. Figure 2.3 and 2.4 show respiratory signs and viral load in tears, respectively, determined 5 and 7 days after challenge in chickens that had been vaccinated at 1-day-old with CEKp7e2. As seen in Figure 2.3, vaccination with either $10^{4.0}$ or $10^{5.0}$ EID_{50} of CEKp7e2 provided significant ($P<0.05$) protection against respiratory signs compared to unvaccinated/challenged birds. No significant differences in respiratory sign scores were detected between chickens vaccinated with the two vaccine doses, indicating that both doses protected against respiratory signs equally well. Consistent with this reduction of clinical signs, a reduction of viral loads was observed in tear fluids of vaccinated birds (Figure 2.4). Significantly higher viral loads were detected in non-vaccinated/challenged chickens both at Days 5 and 7 compared to vaccinated birds. However, on Day 5 a significantly greater reduction of viral load was observed in birds vaccinated with the higher dose of $10^{5.0}$ EID_{50} compared to those vaccinated with $10^{4.0}$ of CEKp7e2. On Day 7 post-challenge this difference had disappeared.

Detection of IBV-specific IgA-secreting cells in Harderian gland lymphocytes by ELISPOT is shown in Figure 2.5. As seen in this figure, expected trends were observed; i.e. there was a greater B lymphocyte response (secondary response) in chickens that had been vaccinated and subsequently challenged, compared to the primary response resulting only from challenge in non-vaccinated/challenged chickens.

The protection results of Trial 2 are shown in Figure 2.6. As seen in this figure, chickens vaccinated with CEKp7e2 as well as chickens vaccinated with the commercial ArkDPI-type vaccine were protected against respiratory signs and showed reduced viral loads compared to unvaccinated/challenged chickens. Significantly lower viral loads were found in tears of chickens vaccinated with the commercial vaccine compared to chickens vaccinated with either dose of the CEK-adapted vaccine. However, protection as determined by evaluation of respiratory signs did not differ significantly among the vaccinated groups. The most similar levels of protection against respiratory signs were detected in chickens vaccinated with $10^{5.0}$ EID_{50} of CEKp7e2 and the commercial vaccine applied at a dose of $10^{4.0}$ EID_{50}.

Protection against emergence of IBV subpopulations. IBV subpopulations recovered 5 days after challenge from tears of chickens that had been vaccinated on Day 1 with either the commercial Ark or the CEKp7e1 vaccine are shown in Table 2.1. The first row on this table shows amino acids in the predominant population based on the S1 sequence of the Ark challenge strain. The following rows show differences to the Ark challenge virus of deduced S1 aa sequences found in challenged chickens, and the number of chickens in which each sequence was found. IBV detected in all non-vaccinated/challenged chickens had S1 sequences identical to the challenge virus. IBV recovered from almost all chickens vaccinated with CEKp7e2 also had S1 sequences identical to the challenge virus. In contrast, subpopulations differing from the predominant population of the challenge virus predominated or were readily apparent in 10 out of 13 chickens vaccinated with the commercial ArkDPI vaccine.

2.5. Discussion

IBV evolves by natural selection, i.e., generation of genetic diversity followed by selection of the most fit phenotypes in the environment (144). ArkDPI-derived vaccines from different manufacturers all show remarkable genetic heterogeneity (104, 151). Selective pressure from the host environment during vaccine virus replication in chickens results in dramatic changes of the predominant population (55, 104, 151). We previously demonstrated that adaptation of an embryo-attenuated IBV ArkDPI-derived vaccine to CEK cells shifted the virus population towards homogeneity in S and NSP genes, and the changes achieved in the S gene in CEK-adapted virus were maintained after one back passage in ECE or chickens (63). However, further selective pressure needed to be applied to the CEK-adapted virus to confirm the stability of those changes, particularly because poultry vaccine production is most cost effective and efficient when virus replication can be performed in ECE. The current sequencing results showed that the changes achieved during CEK adaptation remained after five back passages in ECE. Moreover, deep sequencing results show that at almost all aa positions previously showing selection in CEK cells, homogeneity was either maintained or even increased. Thus it appears that although embryo adaptation of ArkDPI from an unknown number of greater than 50 (58) ECE passages performed in the industry did not stabilize this virus, additional kidney-cell adaptation presented a selective bottleneck narrow enough to increase the stability of this vaccine virus population drastically. Although the biological significance of the different frequencies of aa at one position in NSP2 and three positions in S1 after the second back passage in ECE that had not been altered during CEK adaptation remains unknown, the fact that none of these positions in S1 is located in regions known to contribute to neutralizing epitopes (83, 108) suggests that the ability of the CEK-adapted vaccine to elicit neutralizing antibodies would not be affected. Corroborating previous results (64),

CEKp7e2 conferred effective protection against Ark virulent challenge as determined both by clinical signs and viral load in two animal trials. Furthermore, a strong secondary B lymphocyte specific immune response in the Harderian gland was successfully elicited by the experimental vaccine. In both animal trials a dose effect was detected when using $10^{4.0}$ or $10^{5.0}$ EID_{50} of CEKp7e2. The higher dose provided slightly better results when protection was assessed 5 days after challenge. Because this difference was no longer detected at 7 days post-challenge, it is difficult to ascertain if this difference would be relevant in commercial poultry settings. However, achieving $10^{5.0}$ EID_{50} per dose during poultry vaccine production does not pose a challenge.

A potential concern regarding the CEK-adapted IBV vaccine strain is that changes in S1 occurring during CEK adaptation have altered neutralizing epitopes. Of the three differences in S1 observed, two (at aa 323 and 398) are in regions where neutralizing epitopes have been mapped, each at an aa immediately adjacent to one demonstrated to affect a neutralizing epitope in D107 (83). However, the changes in CEKp7e2 result in identity to the challenge strain at these positions. Thus, potential alterations in neutralizing epitopes due to these changes are not expected to decrease protection by CEKp7e2.

Another potential concern regarding use of a CEK-adapted IBV vaccine strain is that it might be nephrotropic. However, recombinant S protein representing the CEK-adapted vaccine virus does not bind detectably to kidney tissues (150). In addition, multiple vaccine trials have confirmed absence of adverse effects to kidneys in chickens vaccinated with the CEK-adapted vaccine.

This study showed that the changes in the CEK-adapted embryo-attenuated vaccine strain were stable over at least five back passages in ECE. Our previous work demonstrated that the

CEK-adapted vaccine virus is stable during a single back passage in chickens (63). Although we have not examined stability over additional passages in chickens, lack of selection of subpopulations similar to virulent ArkDPI during a single passage in chickens indicates a marked improvement over commercial ArkDPI-derived vaccines, from which subpopulations with S1 sequences similar to virulent ArkDPI strains are selected during a single passage in chickens (55, 104, 111, 112, 151). Furthermore, the vaccine subpopulations with S1sequences similar to virulent ArkDPI were eliminated in the CEK-adapted vaccine or reduced to levels undetectable by deep sequencing (63), reducing the chances of selection of such subpopulations during back passage in chickens.

Besides the fact that typical ArkDPI vaccine subpopulations do not emerge after vaccination with the more homogeneous CEKp7 as previously demonstrated, the most promising result confirmed in this study was that CEKp7e2 vaccination drastically reduces the emergence of subpopulations from the Ark challenge strain (64). Although sequences slightly differing from the challenge virus were detected in only 7%-8% (1/15 and 1/13) of chickens that had been vaccinated with either 10^4 or 10^5 EID_{50} of CEKp7e2, sequences differing from the challenge strain were detected in 77% (10/13) of chickens vaccinated with the commercial attenuated ArkDPI vaccine. This effect could be explained by reduced or even absence of competition between more and less fit subpopulations (e.g., subpopulations showing varying affinity for host cell receptors) found in the CEK-adapted vaccine, which allows this homogeneous population to replicate effectively and thus induce a more specific immune response. Another possible explanation would be that subpopulations in the commercial vaccine-vaccinated chickens are persisting vaccine subpopulations. These subpopulations are not present in the CEK-adapted vaccine, so they are not found in the chickens vaccinated with this virus. We previously compared the effectiveness of

commercial ArkDPI-derived vaccines differing in the proportion of subpopulations selected in chickens prior to selection in the host against Ark virulent challenge (111). Vaccinated chickens were protected against respiratory signs, but subpopulations differing from the predominant population of the challenge strain were selected and became predominant.

Therefore, the homogeneous CEK-adapted IBV ArkDPI vaccine offers improvement and refinement of current ArkDPI-derived vaccines by both eliminating emergence of vaccine-like viruses after vaccination and eliminating emergence of novel strains originating from Ark challenge.

Table 2.1. Deduced aa sequences of the S1 glycoprotein recovered from chickens after Ark virulent challenge. Chickens had been vaccinated on day 1 of age with either increasing dose (10^4 or 10^5 EID_{50}) of CEKp7e2 or with 10^4 EID_{50} of a commercial attenuated ArkDPI-derived vaccine.

		nt	127	176	187	212	226	280-281	283-285	344	385	431	478	488	511	637	665	667
		aa	43	59	63	71	76	94	95	115	129	144	160	163	171	213	222	223
Ark challenge strain			**H**	**A**	**P**	**G**	**F**	**A**	**S**	**F**	**P**	**T**	**P**	**R**	**H**	**A**	**A**	**H**
Group	Number chickens																	
Nv/ ARK	15/15		.[A]	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CEKp7e2-10^4	12/13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CEKp7e2-10^4	1/13		.	.	.	.	.	.	.	.	S	.	.	.	.	.	.	.
CEKp7e2-10^5	14/15		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
CEKp7e2-10^5	1/15		H/Y[B]	.	.	.	F/L	.	S/N	F/Y	.	T/M	.	R/I	H/Y	A/S	A>V[C]	.
Cv[D]	3/13		.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cv	1/13		.	.	.	.	F>L	.	S>N	F>Y	.	T>M	.	.	H>Y	.	.	.
Cv	2/13		.	.	.	.	F/L	A/V	S/N	F/Y	.	T/M	.	.	H/Y	.	.	.
Cv	1/13		.	.	.	.	.	A/V	N	Y	.	M	.	.	Y	.	.	.
Cv	1/13		.	.	.	.	L	V	N	Y	.	M	.	.	Y	.	.	H>Y
Cv	1/13		.	.	S/P	.	L>F	V>A	N>S	Y>F	.	M>T	.	.	Y>H	.	.	.
Cv	1/13		.	.	.	V	.	.	.	.	.	.	.	.	.	.	.	.
Cv	1/13		.	V	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Cv	1/13		.	.	.	.	.	.	.	.	.	.	S	.	.	.	.	.
Cv	1/13		.	.	.	.	L	.	.	.	.	.	.	.	.	.	.	H/Y

[A] Dots indicate amino acid residues matching the Ark challenge S1 sequence.

[B] / = mixed population with amino acid (aa) at similar proportion

[C] > = mixed population with first aa at a greater proportion

[D] Cv = Commercial vaccine

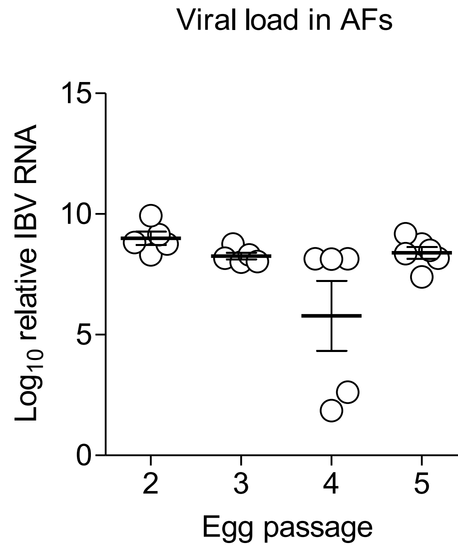

Figure 2.1. IBV RNA levels determined by qRT-PCR in allantoic fluids (AF) during back-passages in embryonated chicken eggs (ECE). RNA was extracted from AFs of five ECE 3 days after inoculation. Individual values, mean and SEM are shown.

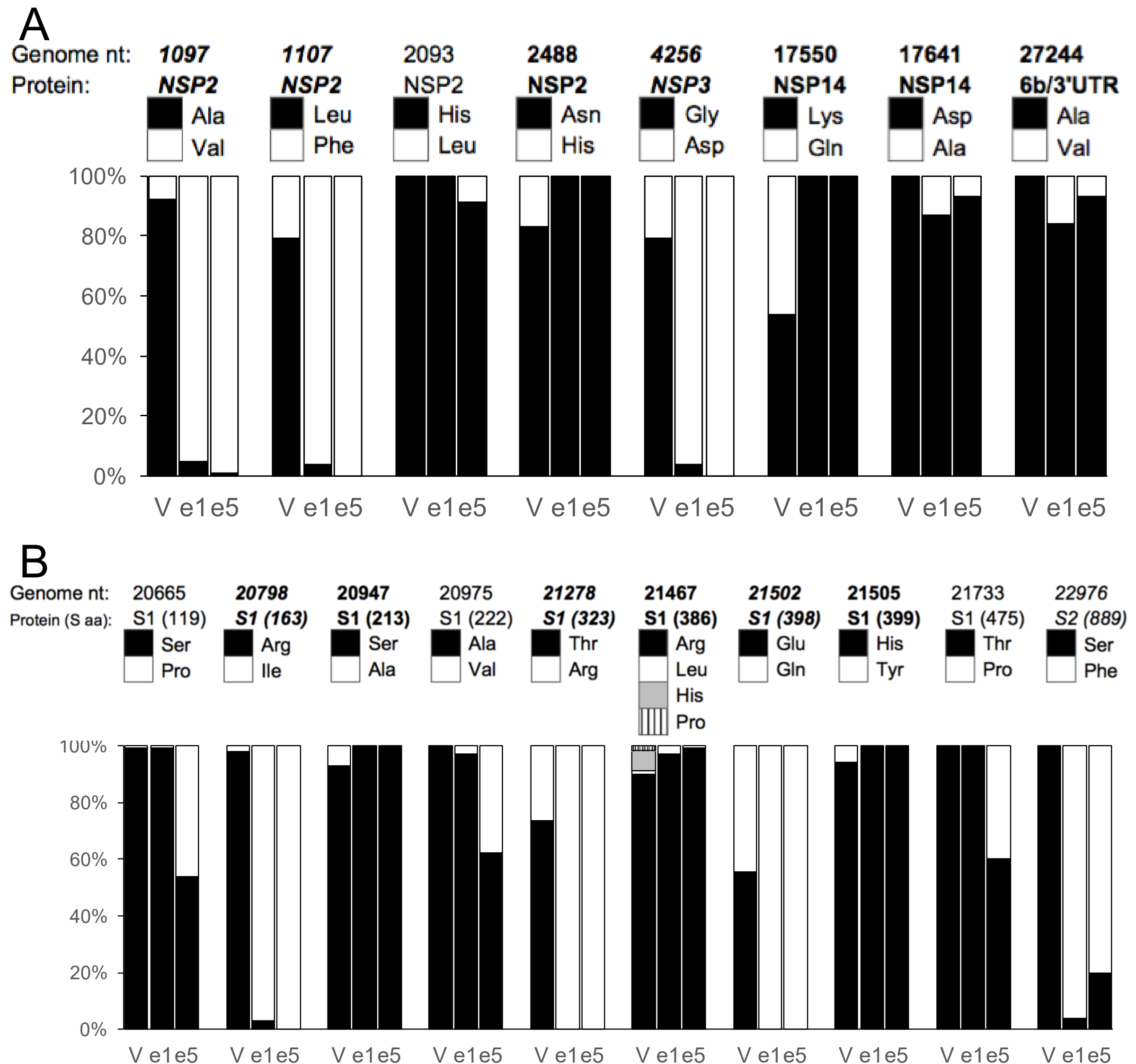

Figure 2.2 . Differences in deduced aa frequencies in NSP (A) and S (B) proteins of CEK-adapted ArkDPI-derived vaccine after five embryo back-passages (e5) compared to previously reported (7) frequencies in commercial vaccine (V) and after one embryo back-passage (e1) determined by next generation sequencing. Positions indicated in Italics are those where the major aa changed during adaptation to CEK cells; bold italics indicate positions where the major aa change was maintained, and increased homogeneity of CEKp7e1 was maintained or further increased during

further back-passages in ECE; bold indicates positions where homogeneity of CEKp7e1 was increased or maintained during further back-passages in ECE; plain lettering indicates positions where the major aa encoded did not change during either CEK-adaptation or back-passages in ECE, but heterogeneity increased during back-passages in ECE. Genome position 22976 (in S2 gene) is the only position at which the major aa encoded changed during adaptation to CEK cells but heterogeneity was increased during back-passages in ECE; however, the change in the major aa encoded was maintained.

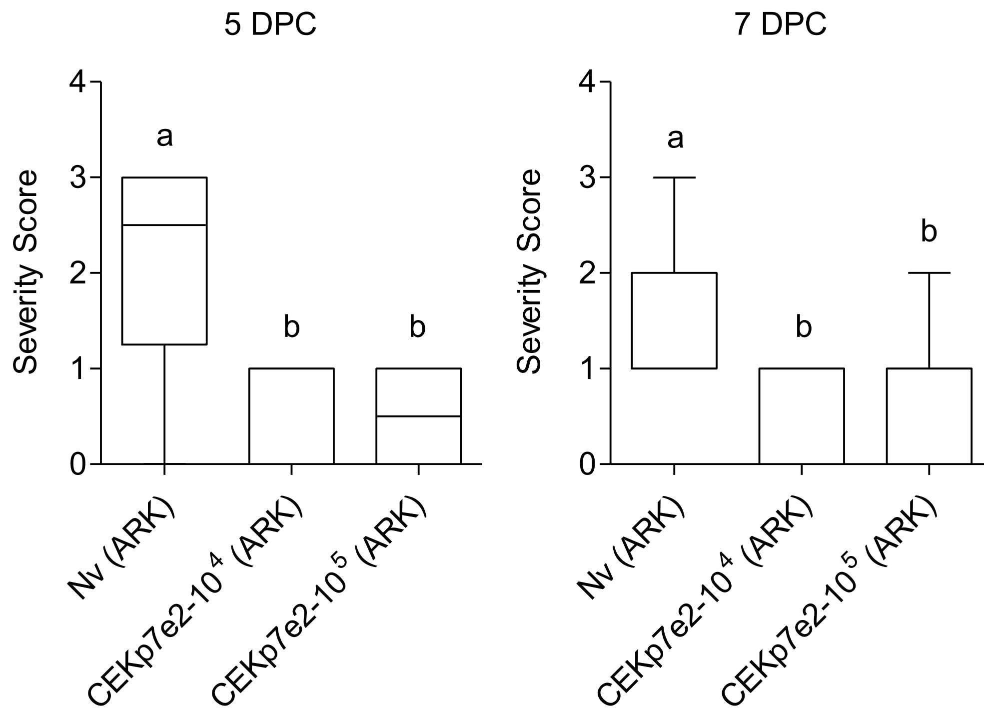

Figure 2.3. Respiratory signs (boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum) 5 and 7 days post-challenge (DPC) with virulent Ark in chickens (n = 12-16/group) vaccinated with CEKp7e2. Vaccination performed on day 1 of age with 10^4 or 10^5 EID_{50}/bird. Ark challenge performed at 20 days of age with 10^6 EID_{50}/bird. Nv (ARK) = unvaccinated/Ark challenged. Different letters indicate significant differences between groups (P<0.05) by Kruskal-Wallis and Dunn's post-test.

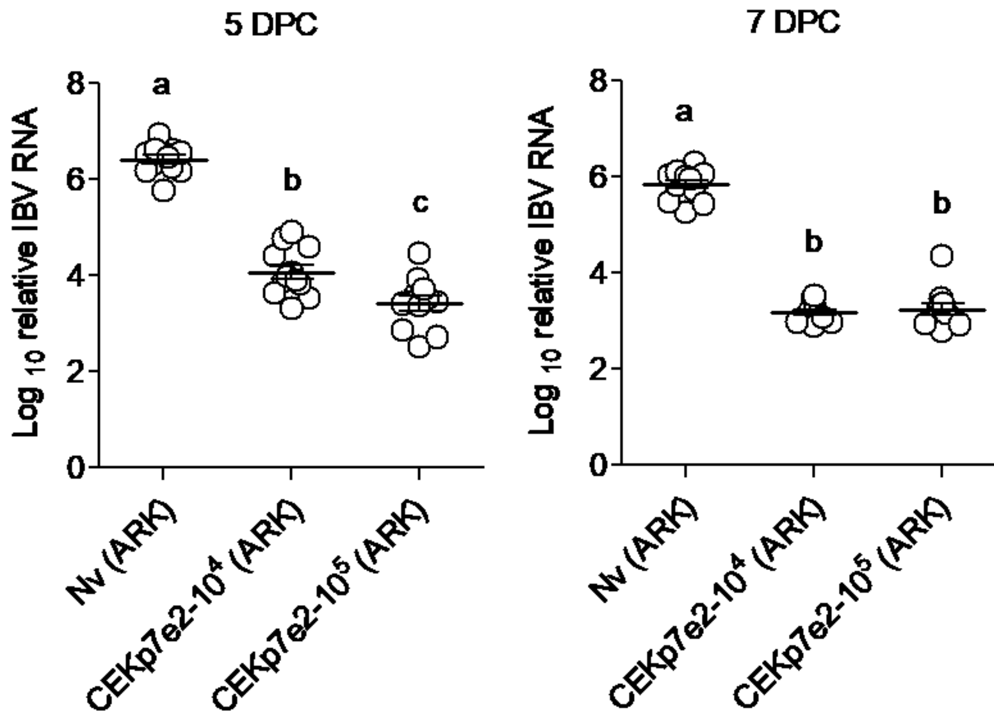

Figure 2.4. IBV RNA quantitated by RT-PCR in tears of individual chickens 5 and 7 days post-challenge (DPC) with virulent Ark in chickens (n = 12-16/group) vaccinated with CEKp7e2. Vaccination and challenge performed as described in Figure 2.3 legend. Nv (ARK) = unvaccinated/Ark challenged. Different letters indicate significant differences between groups ($P<0.05$) by ANOVA and Tukey's post-test.

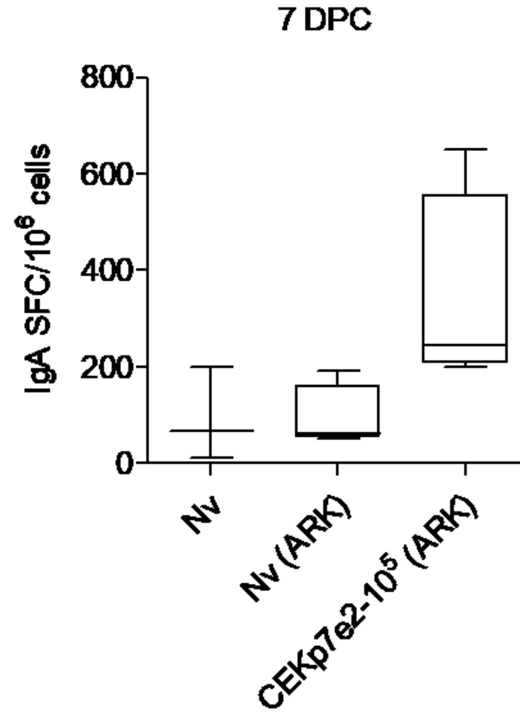

Figure 2.5. B lymphocytes secreting IBV-specific IgA (spot forming cells = SFC) in the Harderian gland (HG) detected by ELISPOT seven days post-challenge (DPC) with virulent Ark in chickens vaccinated with CEKp7e2. Vaccination and challenge performed as described in Figure 2.3 legend. Nv = unvaccinated; (ARK) = Ark challenged. Assays for each group were conducted in four pools of HGs each from 3 chickens. Thus, values for each group represent 12 chickens per group.

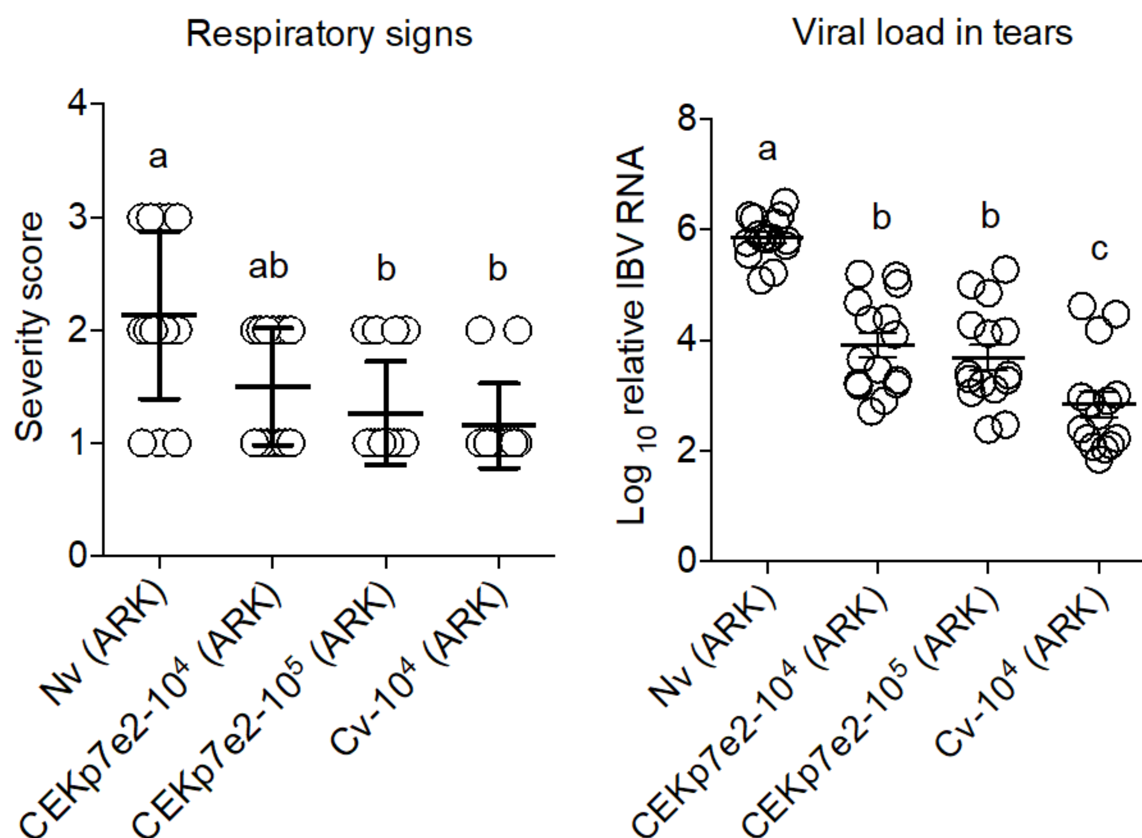

Figure 2.6. Respiratory signs and viral load in tears (IBV RNA by qRT-PCR) on day 5 after Ark challenge in chickens (n = 15 per group) vaccinated on day 1 of age with 10^4 EID_{50} or 10^5 EID_{50} of CEKp7e2 or with 10^4 EID_{50} of ArkDPI-type commercial vaccine (Cv). Challenge performed at 20 days of age with 10^6 EID_{50}/bird of virulent IBV Ark. Nv (ARK) = unvaccinated/Ark challenged. Unvaccinated/non-challenged controls were negative for both signs and IBV RNA (not shown). Different letters indicate significant differences between groups at $P<0.05$.

CHAPTER 3.

Infectious bronchitis virus vaccination at day one of age further limits cross-protection

Avian Diseases (2019) 63: 302-309.

Zegpi, R. A., S. L. Gulley, V. L. van Santen, K. S. Joiner, and H. Toro.

3.1. Summary

Cross-protection and immune responses elicited by infectious bronchitis virus (IBV) vaccination on Day 1 of age or at later time points were examined. Specific-pathogen-free chickens were vaccinated with a Massachusetts-type vaccine and heterologous challenge was performed with an Arkansas (Ark)-type virulent strain. In Trial 1, chickens vaccinated on Day 1 or Day 10 of age were challenged 21 days after vaccination. Analysis of tracheal histopathology and viral load demonstrated less cross protection when vaccination was performed on Day 1 of age. In Trial 2, chickens were vaccinated on Day 1 or Day 14 of age. A somewhat stronger systemic antibody response to IBV was detected in chickens vaccinated at 14 days of age. In addition, avidity of antibodies to Ark-type S1 protein elicited by vaccination at 14 days of age was greater. Differences in immune-cell populations in the Harderian gland (HG) observed at the time of sampling (35 days following vaccination) between chickens vaccinated at 1 day or 14 days of age indicated greater, rather than reduced, immune activity in the chickens vaccinated at 1 day of age. These differences are, perhaps, a result of the higher levels of persisting vaccine virus observed in the younger chickens. Both non-vaccinated/challenged groups showed significantly higher (*P*>0.05) proportions of B cells and $CD8^+$ T cells 7 days after challenge than age-matched vaccinated/challenged groups or age-matched non-vaccinated/non-challenged control groups. These results indicate infiltration and/or expansion of B cells and $CD8^+$ cells in HGs following

challenge of non-vaccinated chickens. A fortuitous finding was that the more immature immune system of chickens vaccinated at 1 day of age was less effective at clearing vaccine virus after vaccination. Collectively, the current results indicate that IBV vaccination at 1 day of age can decrease the potential for heterologous cross protection compared with vaccination at least 10 days after hatch. A lower level of antibody affinity maturation likely contributes to decreased cross protection.

3.2. Introduction

New infectious bronchitis virus (IBV) serotypes/genotypes continuously emerge by mutation and recombination events (144) within the gene encoding the viral spike (S), which mediates viral entry into host cells (15, 26) and is the most variable protein among IBV strains. The S1 subunit of S induces host protective immune responses: virus-neutralizing antibodies (29, 30) , and, along with the viral nucleoprotein (N), cytotoxic T-cell responses that are important in protection against IBV (32). Mucosal immunity, i.e., antibody and T-cell responses at mucosal sites, also play relevant roles in protection against IBV (32, 139, 149). Because of a lack of adequate cross protection between different IBV serotypes, the control of infectious bronchitis (IB) in chicken flocks is routinely addressed with attenuated vaccines covering multiple serotypes. However, despite extensive IBV vaccination, outbreaks of the disease continue to occur. In addition, persistence of vaccine virus in the chickens has been reported, particularly for Arkansas (Ark)-type viruses (75). Besides the biological and evolutionary abilities displayed by IBV enabling them to maintain a high prevalence in poultry flocks, it seems that current vaccination programs beginning on Day 1 of age also contribute to the problem. Indeed, evidence that the immune system of 1-day-old chickens is immature has accumulated since the early 1970s (3, 21, 100, 113, 118, 123, 156, 157). More recently, van Ginkel *et al.* (148) showed that the concentration

of IBV-specific class G immunoglobulin (IgG) in plasma and lachrymal fluid increases significantly if vaccination is performed on Day 14 instead of Day 1 of age. Our most recent results confirmed that levels of both systemic and mucosal IBV-specific antibody responses are highly associated with increasing age of IBV vaccination ($P<0.0001$ and $P = 0.0005$, respectively) (119). Moreover, chickens vaccinated at 1 day of age have shown less protection against homologous IBV challenge than chickens vaccinated on Days 7 or 14 of age (148). The decreased protection was associated not only with reduced antibody responses, but also with decreased IgG avidity when comparing vaccination on Day 1 versus Day 28 of age (148). In the current study, we evaluated the effect of age of IBV vaccination on heterotypic protection. Chickens vaccinated on Day 1 or Day 10 of age with a Massachusetts (Mass)-type vaccine were subsequently challenged with a virulent Ark strain. To understand the mechanisms behind the results of Trial 1, in a second trial we investigated antibody levels, antibody avidity, and Band T-cell responses in chickens similarly vaccinated at 1 day or 14 days of age and subsequently challenged.

3.3. Materials and Methods

Chickens. White leghorn chickens hatched from specific pathogen free (SPF) fertile eggs (Charles River, North Franklin, CT) were maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures were performed in agreement with all federal and institutional animal use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

Vaccine and challenge virus. A commercially available monovalent IBV Mass-type live vaccine (Zoetis, Parsippany, NJ) was used. The IBV Ark-type virulent strain (GenBank Accession No. JN861120) previously described (111) was used for challenge purposes.

Trial 1. Six groups of chickens were established. Groups 1 and 2 (n = 14-15 per group) were vaccinated on Day 1 and Day 10 after hatch, respectively, and were challenged 21 days after vaccination. Two age-matched non-vaccinated/challenged groups (n = 12 per group) and two non-vaccinated/non-challenged groups (n = 10 per group) served as controls. The live attenuated Mass vaccine was administered via the ocular and nasal routes (25 µl in each eye and each nostril) using the dose recommended by the manufacturer. Chickens were challenged via the same route (25 µl into each nostril and each eye) with $10^{6.0}$ 50% egg infective doses (EID_{50}) of the virulent IBV Ark-type strain. Heterologous protection was evaluated 5 days after challenge by relative viral load in tears and tracheal histopathology. Tear samples were collected as described (140) for relative viral load determinations by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Tracheal samples were collected from challenged and control birds 5 days post-challenge for histopathological evaluation.

Relative viral load by qRT-PCR. Relative IBV RNA levels in tears were determined by qRT-PCR. Viral RNA was extracted from individual tear samples using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA). Relative viral RNA concentrations were determined by TaqMan qRT-PCR as described (22). Data were analyzed by one-way analysis of variance (ANOVA) followed by a Tukey post-test. In addition, a limited number of relevant comparisons were evaluated by the Student *t*-test with a Welch correction.

Histopathological evaluation. In brief, formalin-fixed sections of trachea were processed, embedded in paraffin, sectioned at 4-6 µm, and stained with hematoxylin and eosin for histopathological examination. The severity of lesions (tracheal deciliation, epithelial necrosis, mononuclear cell infiltration) was scored (1 = normal, 2 = mild, 3 = moderate, 4 = marked, 5 =

severe) for each chicken. Tracheal lesion scores were analyzed by the Kruskal-Wallis test followed by a Dunn post-test. In addition, a limited number of relevant comparisons were evaluated by the Mann-Whitney test.

Trial 2. In trial 1 we detected enhanced cross protection (see Results section). We hypothesized that the increased protective effect of postponing vaccination would be more notable if a lower challenge dose were used. Thus, in the second trial we reduced the challenge dose by 3.5 log_{10}. In addition, we increased the older age of IBV vaccination and the length of time between vaccination and challenge, as well as the number of control groups. Eight groups of chickens were established (n = 20 per group, except as otherwise noted). Groups 1 and 2 were vaccinated on Day 1 and Day 14 after hatch, respectively, and were challenged 28 days after vaccination. Another four groups were age-matched non-vaccinated/challenged and non-vaccinated/non-challenged (n = 10) controls, as in Trial 1. Two age-matched vaccinated/non-challenged control groups were also included. Challenge was performed with $10^{2.5}$ EID_{50}/bird of the virulent Ark-type strain. Heterologous protection conferred by vaccination was evaluated 5 days after challenge by quantitation of IBV RNA in lacrimal fluids as described for Trial 1. Harderian glands (HG) were collected 35 days after vaccination (7 days post-challenge for challenged groups) from chickens vaccinated on Day 1 and Day 14 after hatch and from non-vaccinated age-matched controls for B- and T-cell determinations (described in the following section). Sera were obtained from vaccinated/non-challenged chicken groups 35 days after vaccination for IBV antibody testing and avidity determination by ELISA (described in a following section).

B and T cells. HGs were collected immediately after euthanasia and placed into cold Ariaans complete medium (7). Cells from five pools, each containing HGs from 4 chickens for each of the

groups containing 20 chickens, were prepared for flow cytometry analysis. For each of the non-vaccinated/non-challenged groups, two pools, each comprised of HGs from four chickens, were analyzed. The HGs were mechanically disrupted by mincing with scissors and forcing them through a stainless steel sieve (provided in the Sigma cell dissociation sieve-tissue grinder kit) with a glass pestle (also provided in the kit). Live mononuclear cells were isolated by density centrifugation over a 1.077-g/ml histopaque density gradient (Sigma Aldrich Corp., St. Louis, MO) at room temperature, and live cells counted using trypan blue exclusion on a Bright-Line hemocytometer (Hausser Scientific, Horsham, PA) as previously described (149). B- and T-cell relative frequencies were determined by flow cytometry essentially as previously described (149). In brief, 10^6 cells were stained using 0.1 µg phycoerythrin (PE) conjugated mouse-anti-chicken Bu-1 monoclonal antibody, PE-conjugated anti-CD44 monoclonal antibody, fluorescein isothiocyanate-conjugated mouse anti-chicken CD8, or biotin-labeled mouse-anti-chicken CD4 and Alexa 660-conjugated streptavidin in a volume of 1 ml. Antibodies were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL), and conjugated streptavidin from Invitrogen (Frederick, MD). All washes were performed with phosphate-buffered saline (PBS) containing 1% bovine serum albumin and 0.1% sodium azide at 4 C. Ghost Dye © red 780 (Tonbo Biosciences, San Diego, CA) was added to exclude dead cells from further analyses. Finally, cells were fixed with a 1% paraformaldehyde solution and stored for analysis the following day. Cell pools were analyzed for B- and T-cell counts in an Accuri© Cytometer using the BD Accuri C6™ software (Accuri© Cytometers, Ann Arbor, MI). A small lymphocyte gate was defined using the controls for each stain and the Ghost Dye© in each sample. Comparisons between groups were performed by ANOVA or Student *t*-test, as appropriate.

ELISA. IBV-specific antibody levels were tested in duplicate by ELISA (IDEXX Laboratories, Inc., Westbrook, ME) in accordance with the manufacturer's recommendations. Because this ELISA uses a Mass serotype virus, to assess the most relevant antibodies, i.e., those recognizing Ark-type S1 (Ark-S1), we also employed an ELISA using recombinant Ark-S1 protein as described (46). Antibody levels (sample to positive [S/P] ratios for anti-IBV antibodies and optical densities [OD] for anti-Ark-S1 antibodies) among groups were analyzed by one-way ANOVA followed by a Tukey post-test. In addition, a limited number of relevant comparisons were evaluated by Student *t*-test with a Welch correction.

Antibody avidity. Antibody avidity (i.e., the accumulated strength of multiple affinities) was determined using a modified ELISA with either Mass-type whole virus coated plates (commercial ELISA plates [IDEXX Laboratories, Inc., Westbrook, ME]) or ELISA plates coated in our laboratory with Ark-S1 recombinant protein. The modified ELISA used increasing dilutions of the chaotropic agent guanidine hydrochloride (GuHCl), which disrupts antibody-antigen interactions but does not alter the integrity of plate-bound antigens (40). Antibody avidity was estimated from the degree to which the GuHCl disrupted the antibody-antigen interaction (40). Sera were diluted 1:100 and added to the commercial IBV ELISA plates or Ark-S1-coated plates. After 30 min of incubation, plates were washed with PBS with Tween20 (PBST) three times and GuHCl dilutions (3, 1.5, 0.75, 0.375, 0.1875, and 0.09375 M in PBST containing 10 mg/ml bovine serum albumin) were added to the wells. After 15 min, plates were washed and the standard ELISA protocol resumed at the step of addition of horseradish peroxidase-conjugated antibody. ODs were determined and data analyzed; a dose-response-inhibitor function [log (inhibitor) vs. response] was used to correlate the logarithm of the concentration of GuHCl to the decrease of OD in the

modified ELISA. The concentration of GuHCl that decreased the OD to 50% of the maximum OD for each sample was calculated and used as an estimator for avidity as described (79).

3.4. Results

3.4.1. Trial 1.

Viral load. Vaccination at either Day 1 or Day 10 of age resulted in a significant reduction in the viral load compared with non-vaccinated challenged controls ($P<0.05$). Significantly lower concentrations of IBV RNA were detected in lacrimal fluids of chickens vaccinated on Day 10 of age after challenge compared with chickens vaccinated on Day 1 of age, whereas viral loads of non-vaccinated birds following challenge were similar in the two age groups (Figure 3.1). Thus, the magnitudes of reduction of challenge viral loads as a result of vaccination, determined by subtraction of the mean $\log_{10}$ viral RNA copies of vaccinated birds from the $\log_{10}$ viral RNA copies of non-vaccinated birds, were higher for birds vaccinated at 10 days of age than for birds vaccinated at 1 day of age ($P<0.05$).

Tracheal damage. The tracheal histopathology findings of Trial 1 are shown in Figure 3.2. Statistically significant reduction in the severity of epithelial necrosis (Figure 3.2 A), tracheal deciliation (Figure 3.2 B), and mononuclear cell infiltration (Figure 3.2 C) scores after challenge of chickens vaccinated at Day 10 of age compared with aged-matched non-vaccinated/challenged controls indicated some degree of protection by vaccination. In contrast, although vaccination at Day 1 of age also resulted in lower tracheal lesion scores following challenge compared with non-vaccinated chickens, these differences were not statistically significant. Furthermore, although tracheal lesion scores were similar in non-vaccinated/challenged chickens in both age groups, epithelial necrosis and deciliation scores of chickens vaccinated at 10 days of age were statistically

significantly lower than those vaccinated at 1 day of age. Mononuclear cell infiltration scores following challenge of chickens vaccinated at 10 days of age were also lower than those of chickens vaccinated at 1 day of age, but the difference was not statistically significant.

3.4.2. Trial 2.

Viral load. In Trial 2 vaccination at either 1 or 14 days of age resulted in a significant reduction in the viral load following challenge compared with non-vaccinated challenged controls ($P<0.05$). Slightly lower IBV RNA levels in tears were found following challenge of chickens vaccinated on Day 14 compared with chickens vaccinated on Day 1, although the differences did not achieve statistical significance (Figure 3.3). A noteworthy fortuitous finding was that non-challenged control chickens vaccinated on Day 1 showed higher concentrations of IBV RNA in tears 33 days post-vaccination than birds vaccinated on Day 14 of age. This indicated that the Mass vaccine persisted at higher levels when administered to younger chickens.

Antibody levels. Antibody levels against whole Mass-type IBV detected by a commercial ELISA kit as well as those against Ark-S1 increased significantly after vaccination (P , 0.05) in chickens vaccinated at either 1 day or 14 days of age compared with non-vaccinated age-matched controls (Figure 3.4). Chickens vaccinated on Day 14 showed somewhat higher IBV-specific antibody levels than chickens vaccinated on Day 1 of age, and analysis of $\log_{10}$-transformed data indicated a statistically significant difference ($P<0.05$). No statistically significant difference was found between ArkS1 antibody levels in chickens vaccinated at different ages.

Antibody avidity. The estimated avidity indices of IBV-specific and Ark-S1 specific antibodies elicited in chickens vaccinated on Day 1 or Day 14 are shown in Figure 3.5. Although the avidity of IBV-specific antibodies estimated using commercial Mass IBV-coated plates was unaffected

by the age of vaccination, Ark-S1-specific antibodies exhibited a significantly higher avidity index (P<0.05) in chickens vaccinated at 14 days of age than in chickens vaccinated at 1 day of age.

B and T cells in HG. Differences were noted between chickens vaccinated at 1 or 14 days of age in immune-cell populations in HGs at the time of sampling 35 days following vaccination; however, these differences were not in the direction expected. Specifically, HGs of chickens vaccinated at 1 day of age had statistically significantly higher (P<0.05) proportions of B cells 35 days following vaccination than age-matched non-vaccinated controls, whereas chickens vaccinated at 14 days of age did not (Figure 3.6 A). In addition, $CD8^+$ T cell proportions were significantly higher (P<0.05) in HGs of chickens vaccinated at 1 day of age than in those of chickens vaccinated at 14 days of age, whereas $CD8^+$ cell proportions did not differ significantly between the two non-vaccinated age-matched control groups (Figure 3.6 C). Similar trends were noted for $CD4^+$ cells, but differences between chickens vaccinated at different ages were not statistically significant (Figure 3.6 B).

Differences in proportions of immune-cell populations in HGs of non-vaccinated/challenged chickens 7 days post-challenge compared with vaccinated/challenged chickens or non-vaccinated/ non-challenged chickens, regardless of age of vaccination, were also noted. In both non-vaccinated/challenged groups, the proportion of B cells was significantly higher (P<0.05) than in age-matched vaccinated/challenged groups or age-matched non-vaccinated/non-challenged control groups (Figure 3.6 A). In addition, $CD4^+$ T cells were significantly increased (P<0.05) in both non-vaccinated/challenged chicken groups compared with their age-matched non-challenged controls (Figure 3.6 B). These results indicate infiltration and/or expansion of B cells and $CD4^+$ cells in HGs following challenge of non-vaccinated chickens, resulting in increased

proportions of these cells 7 days following challenge, which is not observed in vaccinated chickens within the same period following challenge. B cell proportions were greater than the other immune-cell populations evaluated only in non-vaccinated/challenged chickens (Figure 3.6 E). In all other groups, $CD8^{+}$ cells showed the greatest proportion. $CD4^{+}$ cells made up the smallest proportion of immune cells evaluated in all groups (Figure 3.6 E), including non-vaccinated/challenged groups, in which, as already mentioned, they exhibited increased proportions compared with non-challenged controls. Significant differences in the proportion of memory $CD8^{+}$ cells ($CD8^{+}CD44^{+}$) were not noted among chicken groups, although they exhibited trends similar to the other cell types (Figure 3.6 D).

3.5. Discussion.

In this study, we examined cross-protection and immune responses after IBV vaccination on Day 1 of age or at later time points. Vaccination was performed with a Mass-type vaccine virus and heterologous challenge with an Ark-type virulent strain. In Trial 1 chickens were vaccinated on Day 1 or Day 10 of age and subjected to challenge 21 days after vaccination. The analysis of viral load (Figure 3.1) and tracheal histopathology (Figure 3.2) demonstrated reduced heterotypic protection when chickens were vaccinated on Day 1 of age. Previous work showed that postponing vaccination improves protection against homologous challenge (148). The current results expand this knowledge by providing evidence of improved heterologous protection when vaccination is postponed beyond Day 1 of age. We presumed that different ages at vaccination would affect protective responses marginally. Thus, by using a considerably lower challenge dose in Trial 2, we expected greater differences due to age of vaccination. This presumption was not confirmed by the results. Instead, the differences were less notable, as viral loads following challenge were only slightly greater in chickens vaccinated at 1 day of age compared with those vaccinated at 14

days of age, in spite of a somewhat weaker systemic antibody response in the chickens vaccinated at 1 day of age. The weaker antibody response found in chickens vaccinated at 1 day of age corroborates previous reports (119, 148). It also assists explaining the finding that the Mass vaccine virus persisted at higher levels in tears of non-challenged control chickens that were vaccinated on Day 1 compared with levels found in chickens vaccinated on Day 14 of age. This finding suggests that the more immature immune system of very young chickens is less effective at clearing the vaccine virus after vaccination at Day 1 of age. A potential consequence of greater persistence, which ultimately involves increased viral replication cycles, is an increased chance/risk for mutation and recombination events that could possibly result in emergence of virus variants.

In Trial 2 we attempted to find an explanation for the more limited heterologous protection seen when vaccination was carried out at Day 1 of age. Previous work by van Ginkel *et al.* (148) showed greater avidity of whole IBV-specific IgG in chickens vaccinated at 28 days of age than in chickens vaccinated at 1 day of age, and suggested that increased homologous protection following vaccination at increasing ages was associated with increased IgG avidity. In the current study, we did not find a difference between the avidity of whole virus (Mass-type) specific antibodies elicited by vaccination on Day 1 and Day 14 of age. In contrast, we found decreased avidity of the more relevant Ark-S1-specific antibodies in chickens vaccinated at 1 day of age compared with that in chickens vaccinated at 14 days of age. The absence of detectable differences in avidity when using whole virus on the plates could be explained by a masking effect of extensive variation in avidity of a multitude of antibodies directed to a vast number of epitopes that is being determined when using whole virus. In contrast, when using Ark S1 recombinant protein on the plates only the avidity of antibodies directed to regions on the S1 subunit of the spike protein

conserved among Mass and Ark strains are determined. The reduced avidity for Ark-S1 of antibodies in chickens vaccinated at Day 1 of age is consistent with a role for low avidity of antibodies in reduced heterologous protection in chickens vaccinated at 1 day of age.

Cell-mediated immune responses both at systemic and mucosal effector sites have been reported to be age-dependent (97, 100). In the present study, the average proportions of B, $CD8^+$, and $CD8^+CD44^+$ cells were slightly higher in 49- than in 36-day-old unvaccinated/non-challenged chickens (age-matched controls for chickens vaccinated at Day 14 and Day 1 of age, respectively) but the difference was not statistically significant (Figure 3.6 E). The fact that chickens vaccinated at 1 day of age displayed increased rather than decreased, B cell and $CD8^+$ T cells in HGs 35 days post-vaccination compared with chickens vaccinated at 14 days of age may be explained by a continuing immune response to the higher levels of persisting vaccine virus observed in the younger chickens. Statistically significant higher proportions of B cells in vaccinated chickens compared with age-matched non-vaccinated controls were observed only in chickens vaccinated at 1 day of age. We speculate that the absence of statistically significant differences between vaccinated and unvaccinated birds in birds vaccinated at 14 days of age is a result of sampling the birds too long after vaccination. Both non-vaccinated/challenged groups showed significantly higher ($P<0.05$) proportions of B cells and $CD8^+$ T cells 7 days after challenge than age-matched vaccinated/challenged groups or age-matched non-vaccinated/non-challenged control groups (Figure 3.6). These results indicate infiltration and/or expansion of B cells and $CD8^+$ cells in HGs following challenge of non-vaccinated chickens, resulting in increased proportions of these cells 7 days following challenge. T and B-cell responses have been shown to be essential in IBV clearance and protection (32, 124). Expansion of these cells was not observed in HGs of vaccinated chickens within the same period following challenge. This is consistent with previous observations

suggesting a shift of the secondary immune response to IBV from the mucosal to the systemic compartment (67, 119). Perhaps in vaccinated chickens the existing mucosal immunity did not allow the virus to replicate efficiently and generate a booster response at this mucosal site. $CD4^+$ cells made up the smallest proportion of immune cells evaluated in all groups (Figure 3.6 E), including non-vaccinated/challenged groups, in which they exhibited increased proportions compared with non-challenged controls. Significant differences in the proportion of memory $CD8^+$ cells ($CD8^+CD44^+$) were not noted among chicken groups, although they exhibited trends similar to the other cell types (Figure 3.6 D).

Collectively, the current results suggest that the practice of IBV vaccination on Day 1 of age contributes to poor cross protection, and delays vaccine virus clearance. From an applied perspective, these results may be of particular importance in those regions where only Mass-type vaccines are currently licensed, i.e., several South American countries, although further work with other challenge viruses is needed to confirm the findings. Our work examined only immune responses and protection following a single vaccination. We have previously found that lower IBV-specific antibody responses in chickens vaccinated on Day 1 of age can be overcome by booster vaccination (119). Thus, boosting might have the potential to improve cross-protection.

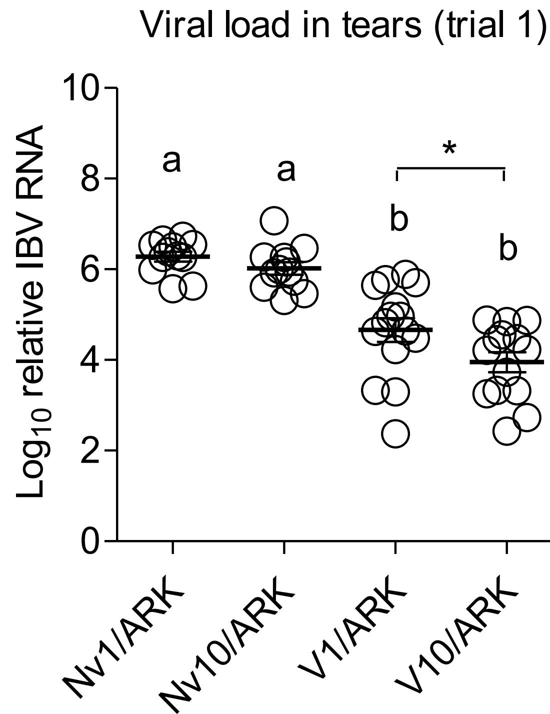

Figure 3.1. Viral load (relative IBV RNA by TaqMan qRT-PCR) in lacrimal fluids 5 days post-challenge in chickens vaccinated with a Mass-type vaccine and subsequently challenged with a virulent Ark-type strain. Chickens vaccinated on Day 1 or 10 post-hatch and challenged 21 days post-vaccination (n = 14-15 for vaccinated groups; n = 12 for non-vaccinated control groups). Nv = non-vaccinated; V = vaccinated; ARK = Ark challenge. Numbers after V indicate the age (days) of vaccination. Numbers after Nv indicate the age of vaccination for which the non-vaccinated chickens are the age-matched controls. Values for each chicken are shown, with means indicated by horizontal lines. Error bars indicate SEM. Different letters indicate significant differences (ANOVA with a Tukey post-test; $P<0.05$). The asterisk indicates additional significant difference demonstrated by the Student *t*-test.

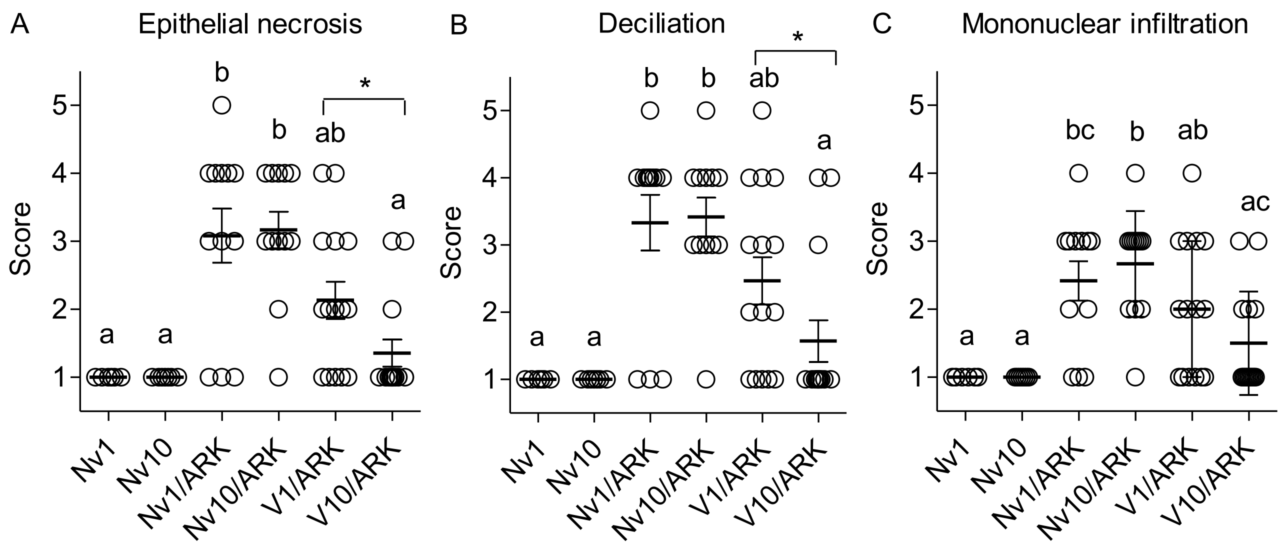

Figure 3.2. Tracheal lesions after virulent Ark challenge in chickens vaccinated with a Mass-type vaccine on Day 1 or 10 of age. Tracheal (A) necrosis, (B) deciliation, and (C) mononuclear cell infiltration scored 5 days after challenge. Nv = non-vaccinated; V = vaccinated; ARK = Ark challenge. Numbers after V indicate the age (days) of vaccination. Numbers after Nv indicate the age of vaccination for which the non-vaccinated chickens are the age-matched controls. n = 5 or 6 for Nv/non-challenged groups, 12 for Nv/ARK groups, and 14 or 15 for V/ARK groups. Lesion scores for each chicken are shown, with means indicated by horizontal lines. Error bars indicate SEM. Different letters indicate significant differences (Kruskal-Wallis test followed by a Dunn post-test; $P<0.05$). The asterisks indicate additional significant differences ($P<0.05$) demonstrated by Mann-Whitney test.

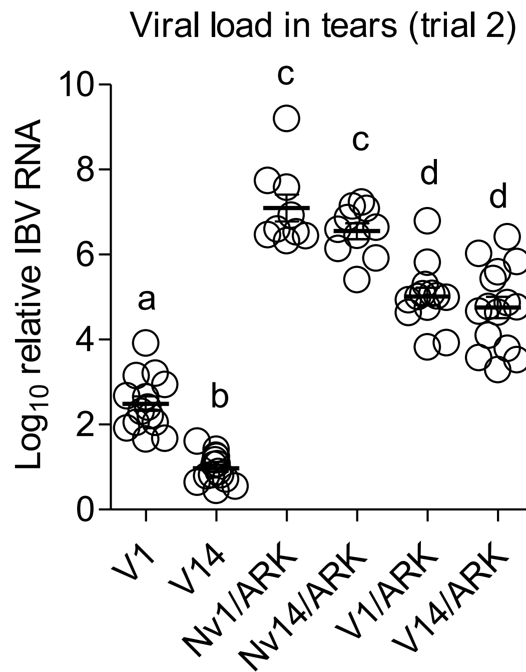

Figure 3.3. Viral load (IBV RNA by TaqMan qRT-PCR) in lacrimal fluids 5 days post-challenge in chickens vaccinated on Day 1 or 14 post-hatch and challenged 28 days post-vaccination. (n = 13-15 for vaccinated/challenged groups, 14-15 for vaccinated/non-challenged groups, and 9-10 for non-vaccinated/challenged groups.) Nv = unvaccinated; V = vaccinated; ARK = Ark challenge. Numbers after V indicate the age (days) of vaccination. Numbers after Nv indicate the age of vaccination for which the unvaccinated chickens are the age-matched controls. Values for each chicken are shown, with means indicated by horizontal lines. Error bars indicate SEM. Different letters indicate significant differences (ANOVA with a Tukey post-test; $P<0.05$).

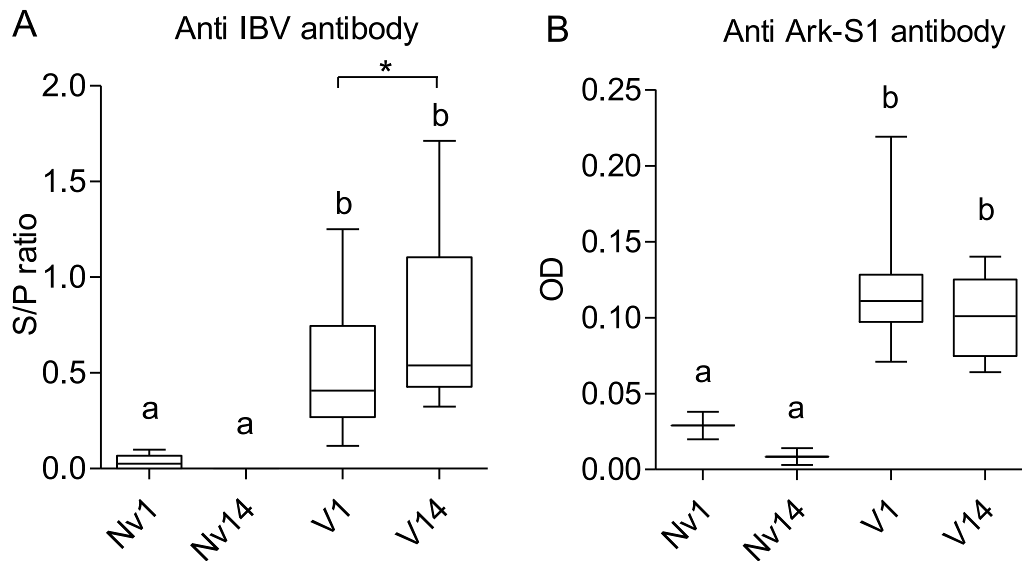

Figure 3.4. IBV (A) antibodies in sera expressed as S/P ratio determined by Mass IBV ELISA and (B) Ark-S1 antibodies determined by recombinant Ark-S1 protein ELISA 35 days after vaccination on Day 1 or 14 post-hatch with a Mass-type vaccine. Nv = non-vaccinated; V = vaccinated; ARK = Ark challenge. Whiskers indicate minimum and maximum values. Different letters indicate significant differences ($P<0.05$; ANOVA with a Tukey post-test). The asterisk indicates an additional significant difference ($P<0.05$) demonstrated by the Student t-test with a Welch correction of log10-transformed data for relevant pairwise comparison.

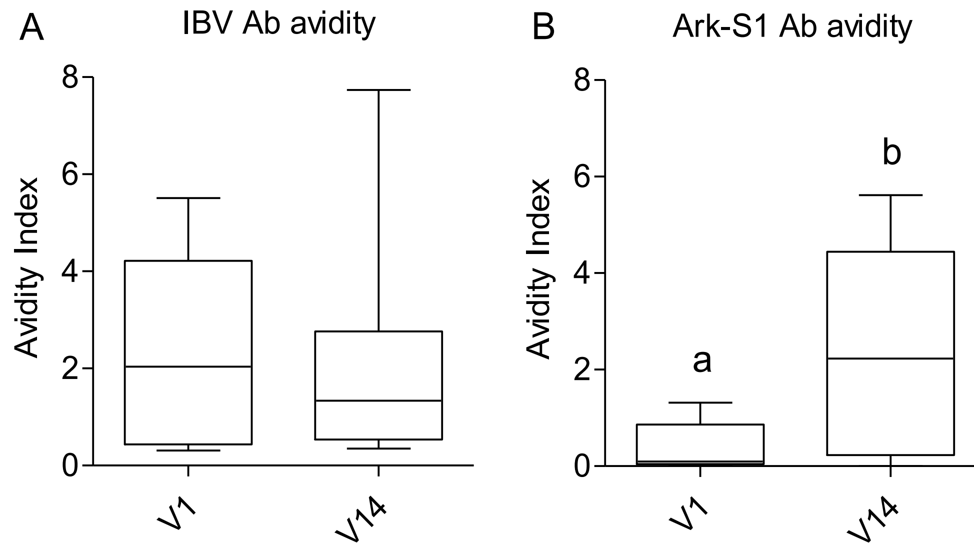

Figure 3.5. Avidity of (A) IBV and (B) Ark-S1 antibodies in sera 35 days after vaccination with a Mass-type vaccine on Days 1 or 14 of age. V = vaccinated. Whiskers indicate minimum and maximum values. Different letters indicate significant differences (*P*<0.05).

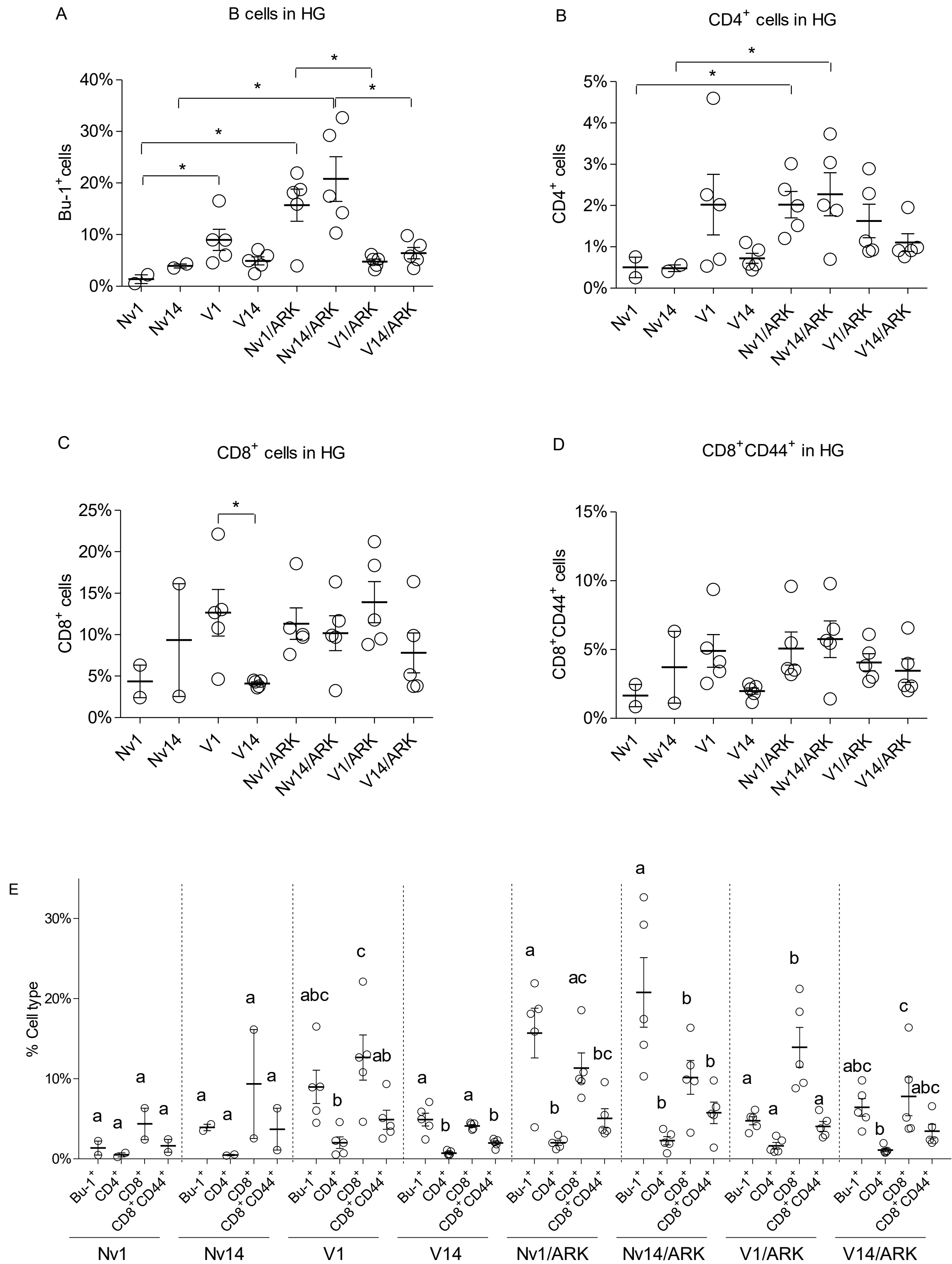

Figure 3.6. Relative frequency of (A) B (Bu-1^{+}), (B) CD4^{+}, (C) CD8^{+}, and (D) CD8^{+}CD44^{+} cells

(expressed as percentage) in HGs of chickens 35 days after vaccination (7 days post-challenge for challenged groups) on Days 1 or 14 of age with a Mass-type vaccine. Nv = non-vaccinated; V = vaccinated; ARK = Ark challenge. Values determined for five pools of Harderian glands from four chickens each are shown, with means indicated by horizontal lines. Error bars indicate SEM. In (A) asterisks indicate $P<0.05$ (both the Student t-test and ANOVA plus a Tukey post-test) for relevant comparisons. In (B) and (C) asterisks indicate $P<0.05$ (Student t-test) for relevant comparisons. The same data for each cell type are presented together in (E) to allow comparison of relative frequencies among cell types in each group of chickens. Different letters indicate statistically significant differences ($P<0.05$; ANOVA plus a Tukey post-test) among cell types within each group of chickens

CHAPTER 4.

Infectious bronchitis virus population structure defines immune response and protection

Avian Diseases (submitted August 2019)

R. A. Zegpi, K. S. Joiner, V. L. van Santen, H. Toro

4.1. Summary

A commercial Arkansas (Ark) Delmarva Poultry Industry (DPI)-type vaccine and a more homogeneous population of that vaccine obtained previously through adaptation to chicken embryo kidney (CEK) cells (CEK-ArkDPI) were used as a model to further understand the impact of population genetic structure on generation of immune responses and protection. In a first experiment, vaccinated chickens were challenged with an IBV Ark99-type virulent strain (AL4614/98). Despite extensive sequence similarity between the vaccines, the more heterogeneous commercial ArkDPI was more efficient at reducing viral loads in challenged chickens, while respiratory signs and tracheal lesions were reduced similarly by either vaccine. A distinct subpopulation of the Ark challenge virus showing asparagine at S1 position 56 was consistently negatively selected by immune pressure originating from vaccination with either vaccine. Antibody levels and antibody avidity to Ark-type S1 protein were greater in CEK-ArkDPI-vaccinated chickens compared to chickens vaccinated with the more diverse commercial ArkDPI vaccine. Synchronous replication of a homogeneous virus population likely elicits clonal expansion and affinity maturation of a greater number of responding B cells compared to a diverse virus population continuously changing its proportion of phenotypes during replication. The results of a second experiment showed that during initial vaccine virus replication (24 and 48 hrs post-vaccination), the virus population showing increased diversity (commercial ArkDPI)

achieved higher concentrations of IBV RNA in the trachea compared to the more homogenous virus. mRNA expression of genes associated with immune responses in the trachea 48 hrs post-vaccination generally showed greater upregulation in chickens vaccinated with the heterogeneous commercial ArkDPI vaccine compared to the CEK-adapted virus. The greater upregulation of these genes is likely associated with higher virus replication achieved by the heterogeneous commercial vaccine. Thus, while the adaptive antibody response was favored by the more homogenous structure of the CEK-ArkDPI vaccine population (higher antibody levels and antibody avidity), the innate immune response was favored by the more diverse viral population of the commercial ArkDPI. We confirmed previous results that distinct subpopulations in wild Ark challenge virus become selected by immune pressure originating from vaccination and concluded that the population structure of IBV vaccines impacts innate immune response, antibody avidity, and protection.

4.2. Introduction

Infectious bronchitis virus (IBV) continues to maintain high prevalence in the world's poultry industry. Continuously emerging new virus serotypes/genotypes arising from mutation and recombination events successfully circumvent extensive vaccination programs (144). An accepted dogma in IBV prevention reads "vaccine strains are selected to represent the antigenic spectrum of isolates in a particular country or region" (quote from 12th edition of Diseases of Poultry). In addition, it is widely perceived by poultry practitioners that not all vaccines belonging to the same serotype are equally effective. Thus, it is common practice for example to use different Massachusetts (Mass)-type vaccines in the same region. Commercial Mass type H-120, Ma5 as well as other Mass vaccines are used in different combinations in search for the most reliable protection. These Mass vaccines differ in strain of origin and method of development (e.g.

attenuation, clonal selection). In the U.S. all currently available Arkansas (Ark)-type vaccines belong to the Ark Delmarva Poultry Industries (DPI) genotype, and were derived from the same virulent isolate. The ArkDPI IBV strain was initially passaged 50 times in embryonating eggs until virulence was reduced (57). The attenuated stock was then distributed to vaccine companies, who each passaged it further in embryonating eggs to generate the commercial Ark vaccines currently available (J. Gelb, University of Delaware, pers. comm.). Despite their common origin and same method of attenuation, commercially available ArkDPI-derived vaccines differ in population structure (i.e., they display different degrees of genetic heterogeneity, ranging from no apparent heterogeneity to heterogeneity in 20 positions in the spike (S) gene) (111, 151). The S protein of IBV mediates viral entry into host cells and is a relevant target for host immune responses (15, 29, 30). Based on S gene sequencing, we previously identified minor vaccine virus subpopulations that become selected in chickens after vaccination with ArkDPI-derived vaccines (55, 151). The rapid selection of these viral subpopulations post-vaccination suggests they replicate better in chickens than the predominant virus population in the vaccine prior to inoculation. Most interestingly, the incidence of specific subpopulations in tears varied among chickens inoculated with the different Ark vaccines and within the chickens vaccinated with the same ArkDPI vaccine (111). The fact that ArkDPI vaccines show different effectiveness at protecting chickens against challenge (111) is likely associated with differing immune responses resulting from the virus population structure.

In previous studies, we subjected a commercial ArkDPI-derived vaccine to selective pressure by passages in chicken embryo-kidney cells (CEK). As determined by next generation sequencing, the virus population shifted towards homogeneity in spike (S) and nonstructural (NSP) genes during passages in CEK cells. Most interesting was the fact that, unlike the heterogeneous

commercial ArkDPI vaccine, no vaccine virus subpopulations emerged in chickens vaccinated with the more homogeneous CEK-ArkDPI (64). We then compared protection conferred by the commercial ArkDPI and the CEK-ArkDPI against challenge with a virulent Ark-type virus (GenBank accession JN861120) previously characterized (54). In CEK-ArkDPI-vaccinated chickens viral subpopulations different from the challenge virus were detected after challenge in a marginal number (7%-8%) of chickens. In contrast, IBV S1 sequences that differed from the predominant population in the challenge virus were detected after challenge in a large proportion (77%) of chickens vaccinated with the commercial ArkDPI attenuated vaccine (166). Thus, the immune responses elicited by the two vaccines seem to differ in quality and/or quantity.

The commercial ArkDPI vaccine and the CEK-adapted ArkDPI vaccine strains provide an excellent model to attempt further understanding the impact of population genetic structure on generation of immune responses and resulting protection. This study was aimed at comparing protection against challenge, emergence of subpopulations from challenge strain, and analysis of immune responses elicited by either vaccine virus. A virulent Ark-type strain that differed from that previously used both in origin and S sequence was used to compare virus subpopulations emerging after virulent Ark challenge in chickens that were vaccinated with the CEK-adapted vaccine virus to those emerging in chickens vaccinated with a commercial ArkDPI-derived vaccine.

4.3. Materials and Methods

Chickens. White leghorn chickens were hatched from specific pathogen-free (SPF) embryonated eggs (Wayward Acres, Pickens, SC) and maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all applicable federal and institutional animal care and use guidelines. Auburn University College of

Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)-accredited institution.

Viruses. The previously described CEK-adapted ArkDPI vaccine virus (64), which had been back-passaged once in SPF embryonated chicken eggs, was used to vaccinate chickens (64). A commercial embryo-attenuated ArkDPI-derived vaccine, from which the CEK-adapted virus originated, was used for comparison. The previously described IBV Ark-type (Ark99 genotype) virulent strain AL/4614/98 (145) (GenBank accession #DQ458217; 98% nucleotide and 97% amino acid (aa) identity to Ark99 S1) was used for challenge. Viruses were titered in SPF embryonated chicken eggs as accepted (57, 152) with slight modifications previously described (64). In brief, in addition to the presence of macroscopic embryo changes, reduced embryo weight and IBV RNA detection by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (22) in embryo allantoic fluids were used to identify infected embryos for calculation of virus titers. Vaccination and challenge in birds were performed with a total volume of 100 µl (25 µl in each nostril and each eye) of appropriately diluted virus stock.

Experimental design. This work consisted of two experiments. Experiment 1 compared protection conferred by CEK-ArkDPI and the commercial ArkDPI vaccines. It also evaluated subpopulations emerging from the challenge strain in vaccinated chickens and evaluated the quantity and quality of antibody responses. Experiment 2 evaluated vaccine viral load and expression of genes associated with innate immune responses early after vaccination.

4.3.1. Experiment 1.

Protection conferred by CEK-ArkDPI compared to commercial ArkDPI vaccine and antibody responses. Four treatment groups were established (n = 20 chickens/group). Chickens

in groups 1 and 2 were vaccinated on day 14 of age with 10^5 EID_{50}/bird of the CEK-ArkDPI and the commercial ArkDPI vaccine, respectively. Control groups 3 and 4 included unvaccinated/challenged and unvaccinated/non-challenged chickens, respectively. All chickens in groups 1-3 were challenged 22 days post-vaccination with 10^5 EID_{50}/bird of virulent IBV Ark AL/4614/98. Protection against challenge was evaluated by assessment of respiratory signs, tracheal histopathology, and viral load in tears and tracheas 5 days post-challenge. IBV RNA extracted from tear fluid of challenged chickens 5 days post-challenge was used to determine virus subpopulations emerging after challenge (described below). Sera from chickens 22 days post-vaccination were used to determine antibody levels and antibody avidity (described below).

Protection and virus subpopulations emerging from the virulent Ark (AL/4614/98) challenge strain. Protection against challenge was evaluated five days after challenge by assessment of respiratory signs, tracheal histopathology, and viral load in tears and tracheas of challenged chickens. Respiratory rales (nasal and/or tracheal) were evaluated blindly by close listening to each bird and scored as 0 (absent), 1 (mild), 2 (moderate), or 3 (severe) as described (146). Clinical scoring data were analyzed by Kruskal-Wallis test followed by Dunn post-test. Tracheal histopathology and histomorphometry was performed as described below. Relative viral loads in tears and tracheas were determined by relative viral RNA levels measured by qRT-PCR (described below). A portion of RNA extracted from tear fluid of each individual challenged chicken was used for partial S1 gene sequencing.

Tracheal lesions. Tracheal histopathology and histomorphometry were performed as previously described (145). In brief, formalin-fixed sections of trachea were processed, embedded in paraffin, sectioned at 4-6 μm and stained with hematoxylin and eosin for histopathological examination.

The severity of lesions (tracheal deciliation, epithelial necrosis, mononuclear cell infiltration) was scored blindly (1 = normal, 2 = mild, 3 = moderate, 4 = marked, 5 = severe) for each chicken. The tracheal mucosal thickness and the thickness of lymphocytic infiltration were measured using ImageJ (https://imagej.nih.gov/ij/download.html), and the average of five measurements for each chicken calculated. Tracheal lesion scores were analyzed by ANOVA.

Viral load (IBV RNA). IBV RNA to be used for qRT-PCR and S gene sequencing was extracted from tear samples using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Relative viral copies in RNA extracted from tear samples collected from individual chickens were determined by quantification of viral RNA by TaqMan© qRT-PCR as described (22). Viral RNA data were analyzed by one-way ANOVA followed by Tukey multiple comparisons post-test. Differences were considered significant with $P<0.05$.

Sequencing of cDNA generated by RT-PCR. A portion of the S1 gene in IBV RNA contained in tears was converted to cDNA and amplified by RT-PCR using the one-step RT-PCR kit (Qiagen, Valencia, CA). Primers S17F and S18R (55) were used to amplify a portion of the IBV S1 gene (66 nt 5' to start of translation through nt 728). RT-PCR products were visualized by gel green stain (Phoenix Research, Candler, NC) after agarose gel electrophoresis. Amplified cDNA was purified using the QIAquick PCR purification kit (Qiagen, Valencia, CA) and submitted to the Massachusetts General Hospital DNA core facility for sequencing using primers S17F or S18R. The sequence of the first approximately 710 nucleotides of the S1 coding sequences was obtained. Sequences were assembled using Mac Vector 10.6.0 software (Mac Vector Inc., Cary, NC). Sequence chromatograms were examined to identify positions with more than one nucleotide identity.

Serum antibody levels. Ark S1-specific antibody levels in sera were determined using a S1-specific ELISA as previously described (119) with minor modifications. Briefly, soluble trimeric recombinant Ark-type S1 protein (46) was produced in human embryonic kidney (HEK) 293T cells as described (158, 159) and used to coat ELISA plates (Nunc MaxiSorp, San Diego, CA). Each plate was incubated for 12 hrs at 4 C with 100 µl of phosphate-buffered saline (PBS) containing 0.2 µg/ml of recombinant S1 protein in each well. Wells were blocked for 3 h at 4 C with 200 µl/well of PBS containing 1% bovine serum albumin and 0.05% Tween 20. Chicken sera were diluted 1:100 in PBS and incubated in the wells for 30 min at room temperature. All following steps were performed with reagents of a commercial IBV ELISA kit (Idexx Laboratories Inc., Westbrook, ME) following the manufacturer's guidelines.

Serum antibody avidity. Antibody avidity (i.e. the accumulated strength of multiple affinities) was determined using a modified ELISA previously described (167) with ELISA plates coated with Ark-S1 recombinant protein (described above). The modified ELISA uses increasing dilutions of the chaotropic agent guanidine hydrochloride (GuHCl), which disrupts antibody-antigen interactions while not altering the integrity of plate-bound antigens. Sera were diluted 1:100, each added into the first ten wells of two rows of a 96 well plate, and incubated for 30 min. The last two wells of each row were used for the positive and blank controls. Plates were washed with PBS with Tween20 (PBST) three times and GuHCl dilutions (5, 4, 3, 2, 1.5, 1, 0.75, 0.25, 0.1 and 0.01 molar in PBST containing 10 mg/ml bovine serum albumin) were added to the wells. After 15 min, plates were washed, and the standard ELISA protocol resumed at the step of addition of horseradish peroxidase-conjugated antibody. Optical densities (OD) were determined and analyzed using an asymmetrical logistic regression curve to correlate the logarithm of GuHCl

concentration to the decrease of OD. Antibody avidity was estimated from the GuHCl concentration that reduced the OD by 50% as described (79).

4.3.2. Experiment 2.

Expression of genes associated with innate immune responses. Chicken groups (n = 24/group) were vaccinated ocularly with 100 µl containing 10^5 EID_{50} of the CEK-ArkDPI or the commercial ArkDPI vaccines at 7 days of age. Unvaccinated age-matched chickens served as controls. Twelve vaccinated and twelve unvaccinated control chickens were euthanized at 24 and 48 hrs post-vaccination. Relative viral loads in lachrymal fluids and tracheas were determined by relative viral RNA levels measured by qRT-PCR as described above. A portion of the proximal third of the trachea was collected from three chickens per group for mRNA detection as described below.

Tracheal RNA extraction and cDNA synthesis. Total RNA from three chicken tracheas per group was extracted using TriReagent® RNA/DNA/protein isolation reagent (Molecular Research Center, Cincinnati, OH) according to the manufacturer's protocol, omitting the isopropanol precipitation step. Tracheal RNA was quantified by NanoDrop (Thermo Fisher Scientific, Waltham, USA). cDNA was synthesized from 0.5 µg total RNA using random hexamers and oligo-dT primers with the RT^2 First Strand kit (Qiagen, Valencia, CA) following the manufacturer's guidelines. Resulting cDNA was diluted to a total volume of 111 µl and frozen at -20 C until use in the PCR array.

Determination of relative mRNA levels of innate immune genes. The commercially available RT^2 Profiler™ PCR array chicken innate and adaptive immune responses kit (Qiagen, Valencia, CA) was used following the manufacturer's guidelines to analyze immune gene expression in the trachea after IBV vaccination with the CEK-ArkDPI or the commercial ArkDPI vaccines. SYBR

Green PCR conducted with primers in each plate detects cDNA of mRNA from 84 chicken genes associated with immune responses against viruses. Threshold cycles (Ct) estimated by the thermocycler for each sample were loaded into a Microsoft® Excel 2007 (Microsoft Corporation, Redmont, WA) file provided by Qiagen (https://www.qiagen.com/us/resources/resourcedetail?id = b3396407-ecb5-4656-ac5d-5ea7b83a397e&lang = en) and analyses conducted. For each sample in each plate, all mRNA Cts were normalized to a set of five housekeeping genes to allow comparisons between samples and groups. A genomic contamination control, reverse transcription controls, and positive PCR controls included in each plate for each sample were used to validate the results. Gene expression levels presenting at least a two-fold difference compared to the control were considered relevant.

4.4. Results

4.4.1. Experiment 1

Protection conferred by CEK-ArkDPI and commercial ArkDPI vaccines. The results of protection against virulent Ark99 (AL/4614/98) challenge conferred by either vaccine are shown in Figure 4.1 and Figure 4.2. As seen in Figure 4.1, the CEK-ArkDPI and the commercial ArkDPI vaccines both significantly reduced respiratory signs ($P<0.05$) compared to unvaccinated controls, without significant differences detected between them. Both vaccines also significantly reduced viral RNA in the tears and tracheas ($P<0.05$) of challenged chickens. However, the commercial ArkDPI vaccine was more effective at reducing viral loads (IBV RNA) in both tears and tracheas ($P<0.05$) compared to the CEK-ArkDPI vaccine. Consistent with clinical signs and viral load results, tracheal histopathology and histomorphometry (Figure 4.2) showed that both vaccines conferred protection to tracheal epithelial cells. A greater reduction of tracheal lesion scores

(necrosis and deciliation) (P<0.05) was observed in chickens vaccinated with the commercial ArkDPI vaccine compared to the CEK-ArkDPI vaccine, and scores for chickens vaccinated with the commercial vaccine were not statistically significantly higher than unchallenged control chickens. However, reduction in lymphocyte infiltration and mucosal thickness were not statistically significantly different in chickens vaccinated with the two vaccines.

Selection of challenge virus subpopulations in vaccinated chickens. Conventional sequencing of IBV S1 after challenge showed that a change of the predominant nucleotide at position 166 in the S1 gene was observed more frequently in vaccinated compared to unvaccinated chickens. This nucleotide change resulted in a change of the AAT codon encoding asparagine (aa position 56) predominant in the challenge virus stock to a predominant TAT codon encoding tyrosine in most vaccinated chickens (Figure 4.3). In contrast, most of the unvaccinated controls maintained the predominant asparagine codon. Analysis of the sequence chromatogram of the S1 gene of the challenge virus stock revealed the presence of a minor (approximately 18%) tyrosine codon at codon 56. Figure 4.4 shows the percent tyrosine encoded at aa position 56 in all vaccinated and unvaccinated chickens. Many of the samples included a mixture of viruses with asparagine and tyrosine codons. IBV in each of the vaccinated chicken groups showed a statistically significantly higher proportion of tyrosine codon than in the unvaccinated chickens (P<0.0001). The mean proportion of virus with a tyrosine codon at S1 codon 56 in chickens vaccinated with the commercial ArkDPI vaccine (76.7%) was higher than in chickens vaccinated with the CEK-ArkDPI vaccine (66.3%) but without achieving a significant difference. The dispersion of values was lower in commercial ArkDPI-vaccinated chickens [Standard deviation (SD) = 15.8%] versus CEK-ArkDPI-vaccinated chickens (SD = 31.9%).

A comparison of aa substitutions in previously used Ark challenge virus [GenBank accession #JN861120] and currently used Ark virus AL/4614/98 (GenBank accession #DQ458217) selected in vaccinated chickens is shown in Table 4.1. While one consistent change was detected in the AL/4614/98 challenge strain, ten substitutions were detected in the previously used challenge strain (166), including a substitution at position 56.

Serum antibody and antibody avidity. Figure 4.5 shows Ark S1 antibody levels and avidity measured 22 days after vaccination with either the commercial ArkDPI vaccine or the CEK-ArkDPI vaccine. Significantly higher ($P<0.05$) antibody levels and significantly greater antibody avidity ($P<0.05$) were detected in CEK-ArkDPI-vaccinated chickens compared to chickens vaccinated with the diverse commercial ArkDPI vaccine. No correlation between individual chicken antibody level and antibody avidity was detected, i.e., a particular bird with a high antibody level did not necessarily show increased avidity and vice versa (correlation analysis not shown).

4.4.2. Experiment 2

Viral load. Figure 4.6 shows relative IBV RNA levels in tears and tracheas determined by qRT-PCR 24 and 48 hrs after vaccination with either the CEK-ArkDPI or the commercial ArkDPI vaccine. Viral loads in tears increased significantly between 24 and 48 hrs post-vaccination. In contrast, in the trachea viral loads maintained similar levels. In addition, while in tears the viral RNA concentration did not differ between chickens vaccinated with either vaccine at either time point, in the trachea considerably higher copy numbers of IBV RNA were detected at both time points in chickens vaccinated with the commercial ArkDPI vaccine ($P = 0.06$ at 48 hrs post-vaccination).

mRNA expression of genes associated with immune responses. Figure 4.7 shows 23 genes displaying up- or down-regulation compared to unvaccinated controls (genes not exhibiting differences compared to unvaccinated controls not shown). The fact that measurements were performed in only three chickens per group prevented meaningful statistical analyses, but nonetheless clear trends were detected. Indeed, 18 genes showed upregulation in vaccinated chickens. In 14 out of 18 genes, a greater gene upregulation occurred in chickens that had been vaccinated with the commercial ArkDPI vaccine while for the remaining four, upregulation was greater in CEK-ArkDPI-vaccinated chickens. Four genes were downregulated by both vaccines and in three of four cases greater downregulation was detected in CEK-ArkDPI-vaccinated chickens. IL17C showed downregulation only by the CEK-ArkDPI vaccine and IL6 showed upregulation only by the commercial ArkDPI vaccine. It was interesting to notice a substantial upregulation of the myxovirus-resistance (MX1) mRNA by both vaccines. Also interesting to notice was that IFNα and β were downregulated by both Ark vaccine viruses.

4.5. Discussion

The commercial ArkDPI vaccine and the CEK-adapted ArkDPI vaccines are evolutionarily separated only by the selective pressure applied over seven passages in embryonic kidney cells. Thus, most of the differences between them are restricted to more or less heterogeneity at particular nucleotide positions over the whole genome as previously reported (63, 166). The behavior in the host (viral replication and tropism) and immune responses induced by either vaccine can therefore be attributed to the population structure. Another relevant factor that can alter the result of a vaccination/protection experiment is most certainly the challenge strain used. In previous studies and in the current experiment we have performed homologous challenge using virulent IBV strains belonging to the same serotype of the vaccine. In both cases however, the challenge strains used

belong to the Ark99 genotype, which slightly differs from the ArkDPI vaccine genotype. Although both challenge strains belong to the Ark99 genotype, they originate from different regions and show limited genetic differences (currently used Ark: GenBank accession #DQ458217 and previously used Ark: GenBank accession #JN861120).

Despite the extensive similarity between the vaccines tested, the more heterogeneous commercial ArkDPI was more efficient at reducing viral load, and deciliation and necrosis of the tracheal epithelium in challenged chickens while respiratory signs, tracheal lymphocytic infiltration, and mucosal thickness were reduced similarly by either vaccine. A comparable result had been previously observed using the different Ark challenge strain previously described (166). It was surprising to determine that using an Ark challenge strain differing slightly from the Ark challenge strain previously used, asparagine at S1 position 56 was also substituted in vaccinated chickens. However, unlike results after use of a different challenge strain (Table 4.1) where as many as 10 substitutions were observed (166), the aa substitution at aa 56 was the only consistent change seen in the current Ark challenge strain replicating in vaccinated chickens.

Figure 4.8 shows a structural model of the S1 N-terminal domain based on structural homology modeling with M41 S (127). In this model, we have indicated positions showing the aa substitution in challenge strain AL/4614/98 and the positions of aa substitutions in previously used challenge strain in CEK-ArkDPI-vaccinated chickens for comparison. For better understanding of the potential role of immune pressure in the aa substitutions, we have also indicated positions corresponding to aa substitutions of neutralizing monoclonal antibody escape mutants of Mass serotype strains previously determined by others (30, 83), presumably corresponding to parts of neutralizing epitopes. Note that substitutions tend to accumulate at close positions on the same

side of the S1 N-terminal domain. We concluded that, in general, distinct subpopulations in wild Ark challenge virus become selected by immune pressure originating from vaccination. Antibody levels and avidities were measured using recombinant S1 protein-coated plates. Thus, the antibody responses measured do not necessarily represent the responses to the wide range of conserved and less conserved IBV antigenic determinants exposed to the immune system during viral replication, but exclusively epitopes on the S1 protein. It is also relevant to note that the S1 protein used for the current analyses was produced using the sequence of one of the subpopulations of ArkDPI vaccine virus selected in chickens (46). The fact that both antibody levels and antibody avidity were greater in CEK-ArkDPI-vaccinated chickens could be explained by the increased homogeneity of the replicating virus population. We have previously shown that different subpopulations of ArkDPI replicate at different places in the host (111). This is likely associated with differing fitness of distinct phenotypes. Thus, we speculate that because of reduced phenotype diversity, the population of CEK-ArkDPI should replicate more in synchrony as a whole. Synchronous replication of a homogeneous virus population likely elicits clonal expansion and affinity maturation of a greater number of responding B cells compared to a diverse virus population continuously changing its phenotype during replication. The results of experiment 2 showed that initial replication of the vaccine viruses in tears did not differ significantly between the vaccines tested. There was however an increase of viral load between 24 and 48 hrs post-vaccination. This increase was not detected in the trachea at these time points. However, in the trachea higher levels of IBV RNA were detected in birds vaccinated with the commercial ArkDPI vaccine. These higher levels of replication in the trachea are likely associated with the virus population showing increased diversity, which therefore had increased chances of successful binding to tracheal cells. Indeed, previous protein histochemistry work demonstrated enhanced

binding to tracheal epithelium of a recombinant soluble trimeric S representing the predominant population of the commercial ArkDPI vaccine compared to binding by S of the CEK-adapted virus (150). The mRNA expression levels of genes associated with immune responses in the trachea 48 hrs post-vaccination showed that consistently greater upregulation of innate immune genes was triggered by the commercial ArkDPI vaccines compared to the CEK-adapted virus. The greater upregulation of genes is likely associated with higher virus concentrations achieved by the heterogeneous commercial vaccine, which is linked to increased binding to tracheal cells as discussed above. It is interesting that, while the adaptive antibody response was favored by the more homogenous structure of the CEK-ArkDPI vaccine population (higher antibody levels and antibody avidity); the innate immune response was favored by the more diverse viral population of the commercial ArkDPI. As mentioned in the results section the Mx gene was highly upregulated by both IBV vaccines. Chickens have a single Mx gene that is produced by host cells in response to type I interferons. The lower Mx mRNA expression is consistent with the lower expression of interferon in chickens vaccinated with the CEK-adapted vaccine compared to the commercial vaccine. Although at this time we do not know if Mx has any relevant activity against coronaviruses in chickens, Mx antiviral activity was reported against Newcastle disease virus (162) and avian influenza virus (49) . We conclude that, in general, the population structure of IBV vaccines affects innate immune response, antibody avidity, and protection. From an applied perspective, these results explain observations and vaccination practices by poultry practitioners who frequently test same-serotype IBV vaccines from different companies to assess which confers better protection to their flocks.

Table 4.1	Amino acid position										
	56	63	67	76	94	95	115	144	160	171	223
Ark99 AL/4614/98	Asn	Ser	Ala	Phe	Thr	Asn	Tyr	Met	Ser	His	His
Changes in vaccinated/challenged chickens	Tyr										
No. chickens	33/40										
Ark99 challenge	Asn	Pro	Ala	Phe	Ala	Ser	Phe	Thr	Pro	His	His
Changes in vaccinated/challenged chickens	Ser		Val	Leu	Val	Asn	Tyr	Met	Leu/ Ser	Tyr	Tyr
No. chickens	3/43		3/43	12/43	5/43	12/43	12/43	12/43	3/37	12/43	2/43

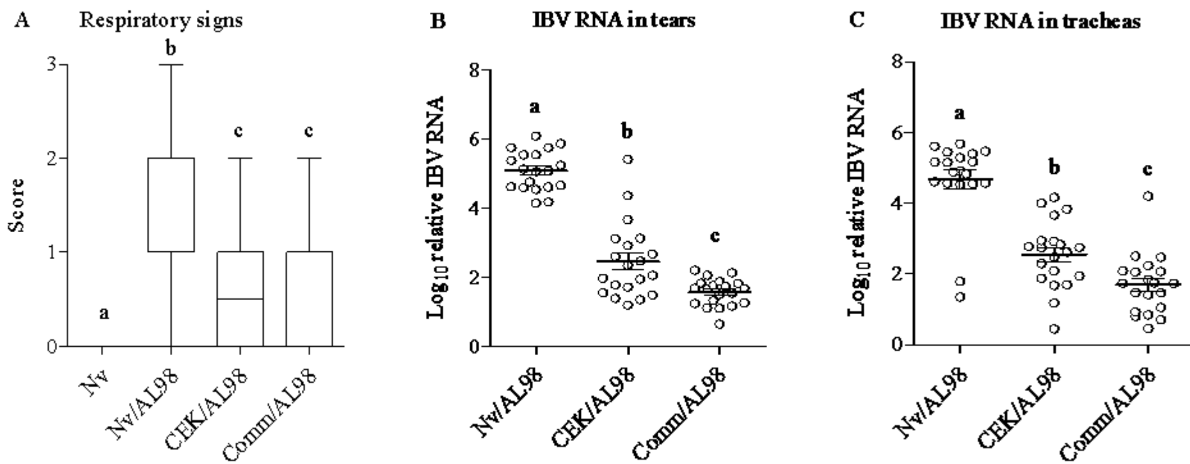

Figure 4.1. Protection conferred by CEK-ArkDPI or commercial ArkDPI against homologous challenge with virulent AL/4614/98 (AL98). Chickens (n = 20/group) vaccinated at 14 days of age with 10^5 EID_{50}/bird and challenged 22 days post-vaccination. Signs and viral loads determined 5 days post-challenge. (A) Respiratory signs (boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum). Different letters indicate significant differences between groups (P<0.05) by Kruskal-Wallis test and Dunn post-test. Relative IBV RNA levels determined by quantitative RT-PCR in tears (B) and tracheas (C) of individual chickens. Values for each chicken are shown, with means indicated by horizontal lines. Error bars indicate SEM. Different letters indicate significant differences (P<0.05) between groups determined by ANOVA and Tukey's post-test. Nv = unvaccinated; Nv/AL98 = unvaccinated challenged; CEK/AL98 = vaccinated with CEK-adapted ArkDPI vaccine and challenged; Comm/AL98 = vaccinated with commercial ArkDPI vaccine and challenged.

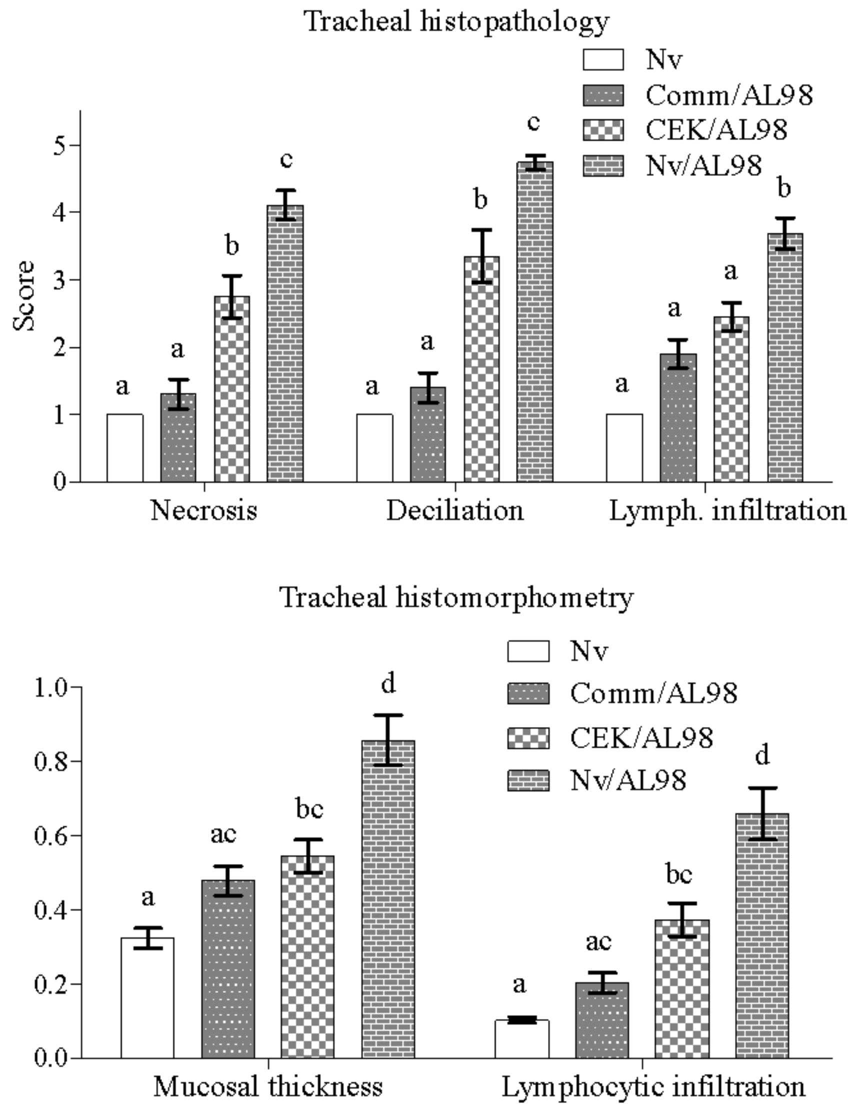

Figure 4.2. Tracheal histopathology and histomorphometry of chickens treated as described in legend of Figure 4.1. Nv = unvaccinated; Nv/AL98 = unvaccinated challenged; CEK/AL98 = vaccinated with CEK-adapted ArkDPI and challenged; Comm/AL98 = vaccinated with commercial ArkDPI and challenged. Differences analyzed by ANOVA. Different letters indicate significant differences ($P<0.05$).

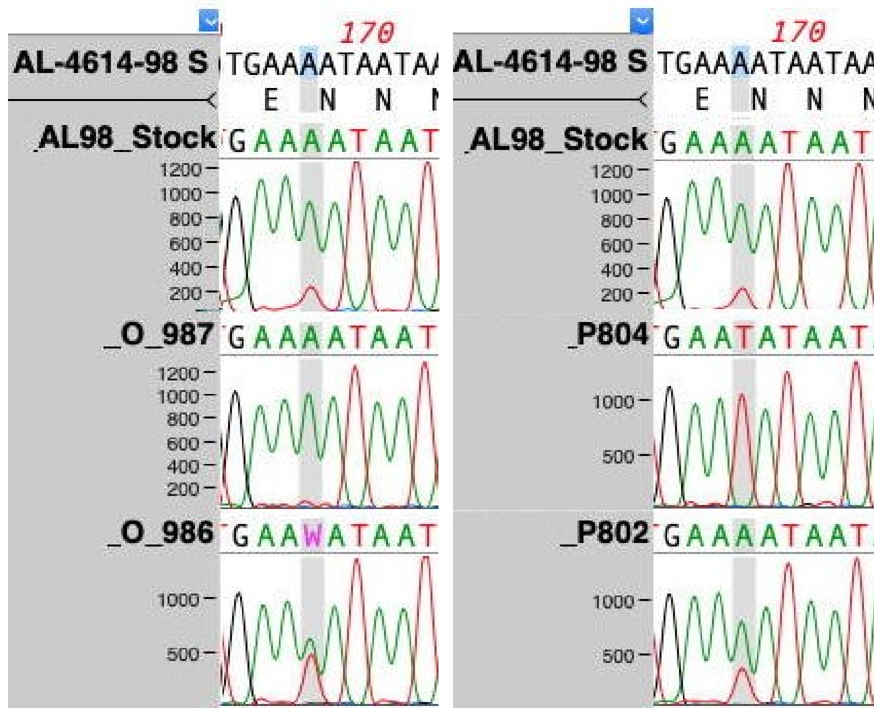

Figure 4.3. Examples of S1 sequence chromatograms of IBV RNA contained in tears from unvaccinated and vaccinated chickens 5 days after challenge. Sequence chromatograms of the AL/4614/98 stock inoculated into chickens are shown at the top for comparison. Results from two unvaccinated chickens (0987 and 0986) and two vaccinated chickens (P804 and P802) are shown. Vaccinated chicken P804 shows a nearly complete change of the AAT codon at position 166 encoding asparagine [amino acid (aa) position 56] to TAT encoding tyrosine as detected in many vaccinated chickens compared to most unvaccinated controls (chicken O987) maintaining the sequence of the challenge strain prior to replication in chickens. Some vaccinated and unvaccinated chickens clearly showed mixed populations (e.g. chickens (O986 and P802). The % tyrosine codon for the challenge virus populations found in tears of each chicken 5 days after challenge are shown in Figure 4.4.

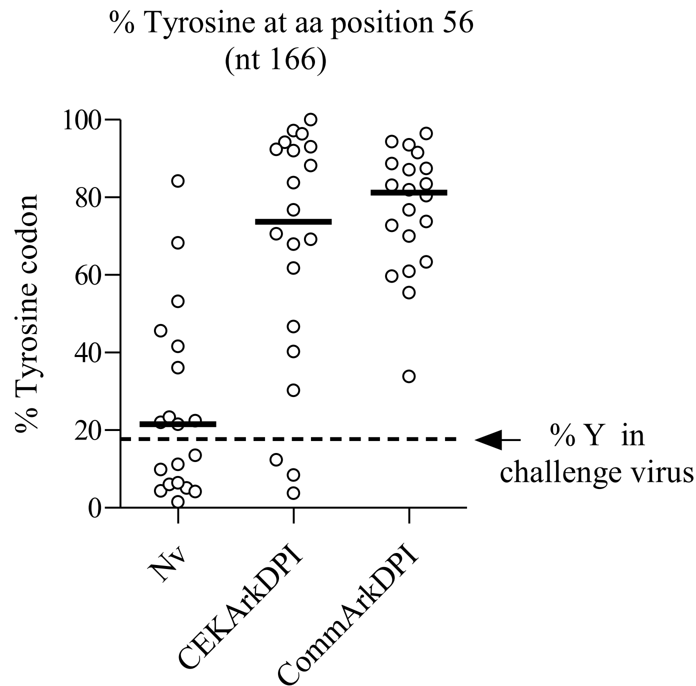

Figure 4.4. Percent tyrosine codon at S1 aa position 56 in IBV RNA in tears of vaccinated and unvaccinated chickens 5 days after challenge with virulent AL/4614/98. Dotted line indicates percent tyrosine (Y) codon in challenge virus stock prior to replication in chickens. Challenge virus in each of the vaccinated chicken groups showed a statistically significantly higher proportion of tyrosine codon than in the unvaccinated chickens ($P<0.0001$). The mean proportion of virus with a tyrosine codon in chickens vaccinated with the commercial ArkDPI vaccine (76.7%) was higher than in chickens vaccinated with the CEK-ArkDPI vaccine (66.3%) but without achieving a significant difference. The dispersion of values was lower in commercial ArkDPI-vaccinated chickens (SD = 15.8%) versus CEK-ArkDPI-vaccinated chickens (SD = 31.9%).

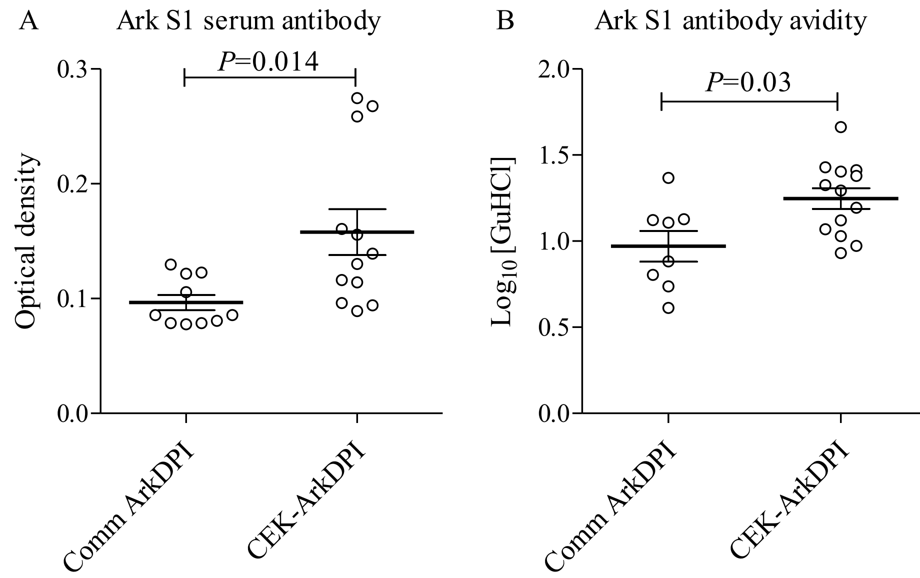

Figure 4.5. Ark S1 antibodies (A) and antibody avidity (B) in sera 22 days after vaccination at 14 days of age with the CEK-adapted ArkDPI (CEK-ArkDPI) or the commercial ArkDPI (Comm ArkDPI) vaccines. *P* values (Student's *t* test) for significant differences are indicated.

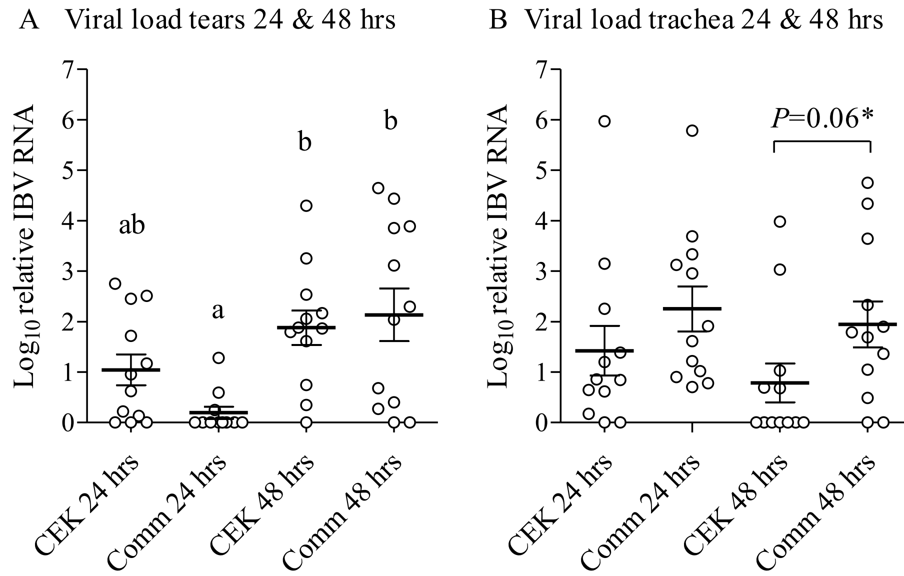

Figure 4.6. Viral load (IBV RNA by TaqMan quantitative RT-PCR) in tears (A) and tracheas (B) 24 and 48 hrs after vaccination with CEK-ArkDPI or commercial ArkDPI. Chickens (n = 12/group) vaccinated on day 7 post-hatch. Values for each chicken are shown, with means indicated by horizontal lines. Error bars indicate SEM. Different letters indicate significant differences (ANOVA with Tukey's post-test; $P<0.05$). *Additional testing by *Student's t* test indicates a difference approaching statistical significance ($P = 0.06$).

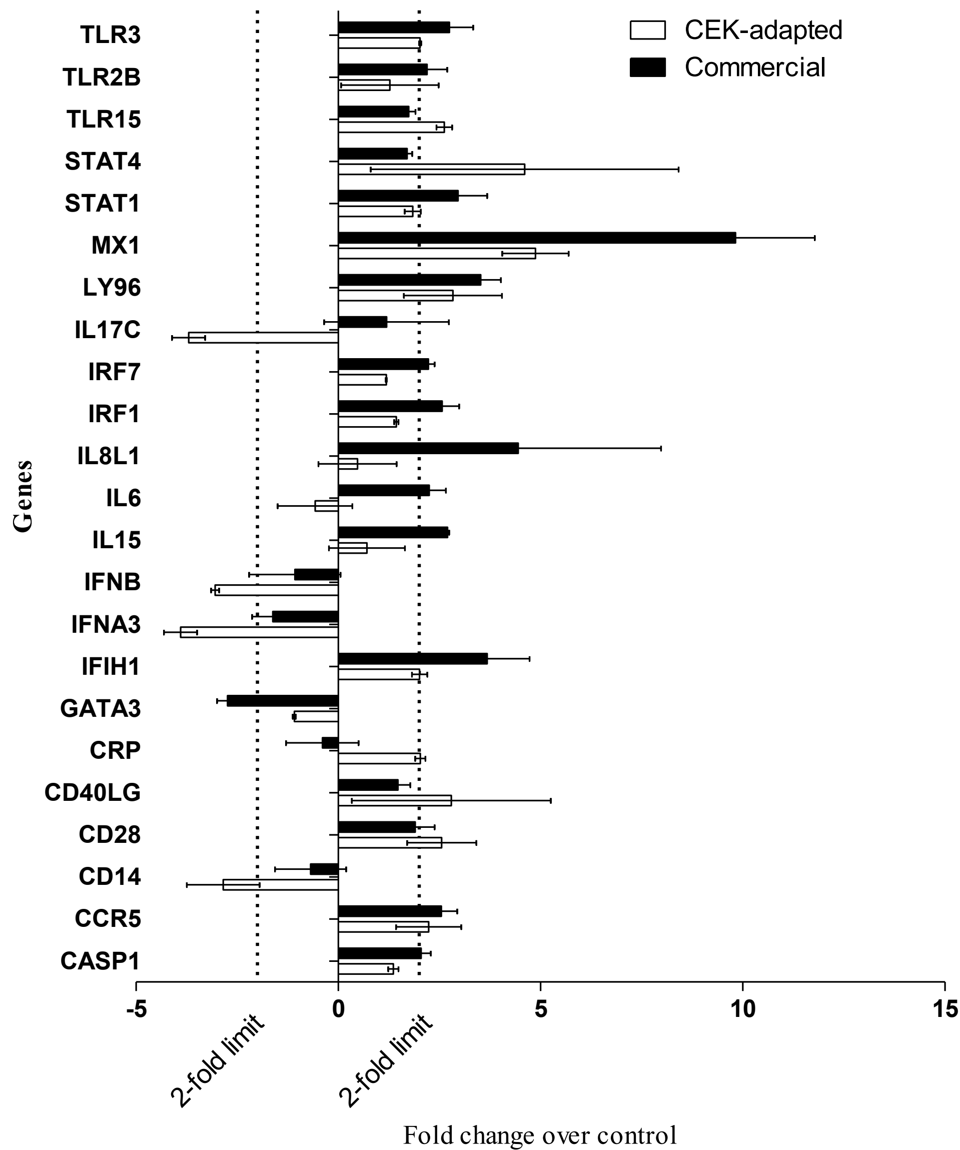

Figure 4.7. mRNA expression of innate immune genes in the trachea of individual chickens (n = 3/group) vaccinated with the CEK-ArkDPI or the commercial ArkDPI vaccines using the RT² Profiler™ PCR array (Qiagen, Valencia, CA). Out of 84 genes tested, 23 genes displayed up- or downregulation (two-fold difference compared to unvaccinated controls). Genes not exhibiting differences compared to unvaccinated controls not shown.

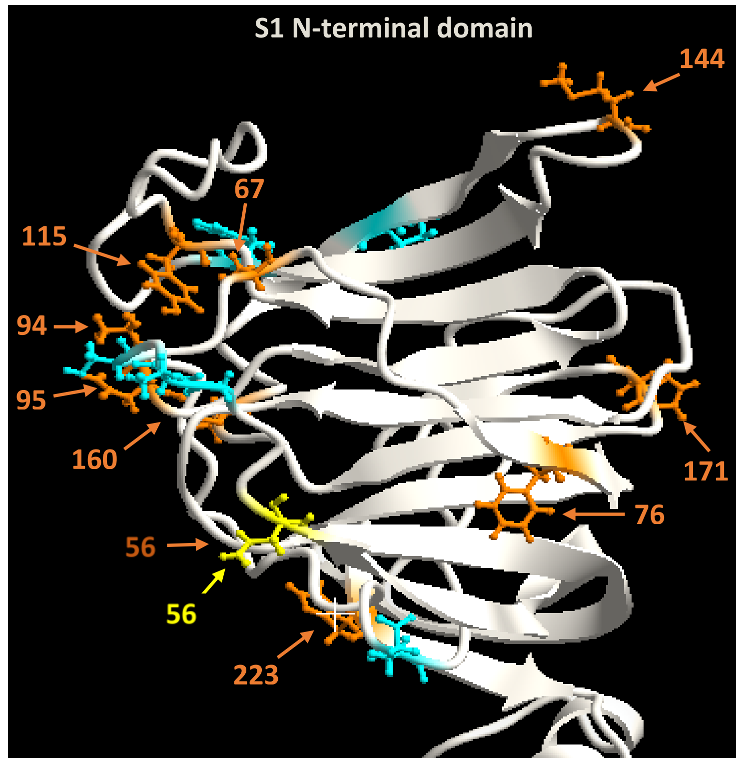

Figure 4.8. Ribbon model of AL/4614/98 S1 N-terminal domain structure generated by I-TASSER structural homology modeling based on M41 S structure. Ball and stick models indicate positions of aa substitutions in AL/4614/98 challenge strain (yellow), aa substitutions in previously used Ark challenge strain (orange), and positions corresponding to aa substitutions of neutralizing monoclonal antibody escape mutants of Mass serotype strains previously determined by others (turquoise). Substitutions tend to accumulate at close positions on the same side of the S1 N-terminal domain.

CHAPTER 5.

Protection conferred by IBV S-ectodomain expressed from recombinant NDV LaSota

Avian Diseases (submitted Sept. 2019)

R. A. Zegpi, Q. Yu, K. S. Joiner, V. L. van Santen, H. Toro

5.1. Summary

A recombinant Newcastle disease virus (NDV) LaSota (LS) expressing secreted trimeric spike (S)-ectodomain of infectious bronchitis virus (IBV) (rLS/IBV.Se) was developed and evaluated for protection conferred against IBV challenge. The IBV S-ectodomain protein, which is S excluding the transmembrane anchor and short cytoplasmic domain of S2, expressed from recombinant LS corresponds to an Arkansas (Ark)-type IBV. In a first experiment, chickens were primed at 1-day of age or primed and boosted with 10^4 EID_{50}/bird of rLS/IBV.Se and challenged with a virulent Ark strain. While single vaccination proved completely ineffective at protecting chickens against challenge, priming and boosting reduced clinical signs and tracheal lesions but did not reduce viral load in lachrymal fluids. In experiment 2, the vaccine dose was increased to 10^7 EID_{50}/bird and a different Ark virulent strain was used for challenge. In addition, chickens were singly immunized on either day 1 or day 10 after hatch. NDV antibody levels detected in vaccinated chickens were moderate with hemagglutination inhibition titers varying between 4 and 5 log_2. Slightly higher antibody levels to NDV were observed in chickens vaccinated on day 10 versus day 1 but without the difference achieving statistical significance. In contrast, antibody responses using recombinant IBV S1 protein-coated ELISA plates were significantly greater in chickens vaccinated on day 10 compared to day 1. The use of a higher rLS/IBV.Se dose proved to enhance the success of single vaccination substantially compared to experiment 1. Signs and

tracheal lesions were reduced more effectively in chickens vaccinated at day 10 after hatch. However, as in experiment 1, vaccination did not reduce the viral loads in tear fluids of challenged chickens. Similar results, in which no reduction in viral load in the trachea was apparent from rLS/IBV.S vaccination, have been obtained by others. Further work is needed to understand the immune responses induced by this recombinant virus that seems to provide some protection against the disease but does not reduce viral loads in the upper respiratory tract.

5.2. Introduction

Infectious bronchitis virus (IBV) is endemic in the United States poultry industry and continues to cause economic losses due to increased condemnations at processing plants and declines in egg production. IBV's success in chickens is due to its population plasticity and unique capability to evolve (144). This viral mechanism efficiently counteracts extensive vaccination programs with serotype-specific attenuated live vaccines. In addition, the increasing number of Arkansas (Ark)-like viruses obtained from Ark-vaccinated chickens in the U.S. suggests not only that some vaccines provide inadequate protection, but also that they may themselves be contributing to the problem. Ark vaccine virus subpopulations, which quickly become predominant in chickens after vaccination (54, 104, 151), likely provide a source for emergence of novel ArkDPI-vaccine-like viruses commonly isolated from broiler respiratory disease cases (76). In addition to subpopulations originating from vaccine virus, accumulating evidence shows that subpopulations emerge from wild Ark challenge virus in chickens that have been vaccinated with attenuated ArkDPI vaccines (63, 111, 166). Thus, current ArkDPI-derived vaccines contribute to emergence of novel Ark-like variants in the industry. Therefore an advantage of use of recombinant vaccines in the poultry industry over the current practice of using varying serotype-specific attenuated vaccines would be to reduce emergence of novel IBV (i.e. vaccine-like viruses

originating from selection of vaccine subpopulations in flocks, as well as recombinants involving vaccine viruses of multiple serotypes or vaccine viruses and circulating viruses).

We previously found that while expression of the avian influenza hemagglutinin gene from replication-defective human adenovirus conferred excellent protection against avian influenza virus (130, 141, 142), expression of different IBV S1 proteins showed suboptimal protection against IBV challenge (146). We also previously developed recombinant Newcastle disease virus (NDV) LaSota (rLS) expressing the IBV S2 gene of an IBV Ark strain and demonstrated protection against IBV challenge (147). However, the level of protection achieved was inconsistent and varied among S2 sequences used for the insert. Suboptimal protection by recombinant virus expressing S1 can now be explained by our recent work comparing vaccination with recombinant S1 and S-ectodomain proteins, i.e. vaccination with S1 protein alone is much less protective than vaccination with S-ectodomain protein (S1 extended by the S2 ectodomain) (46). Thus, vaccination using both S1 and the S2 ectodomain seems to be the most promising vaccine strategy.

The NDV LaSota strain has been used as a live vaccine vector and exhibits several advantages including safety, stability, and suitability for mass-administration by spray or drinking water, as well as strong induction of local and systemic immune responses. Insertion of a foreign gene into the LaSota genome reduces the virus' virulence to the level of the mild NDV B1 vaccine strain so that 1-day-old vaccinated chickens do not show any vaccine side-effects but are protected against NDV challenge (48, 164).

In recent years, other groups have developed and evaluated use of recombinant NDV LaSota expressing the IBV complete S protein (rLS/IBV.S) (1, 128). Vaccination with rLS/IBV.S provides some level of protection but effectiveness is below the levels of conventional attenuated

vaccines. Shirvani *et al.* (128) reports induction of IBV-specific neutralizing antibodies and protection against both clinical signs and virus shedding, as indicated by viral RNA levels in tracheal swabs, following challenge in chickens vaccinated with rLS/IBV.S at 4 weeks of age. Reduction in virus shedding when chickens were vaccinated at 1 day of age was dependent on route of challenge. Only ocular challenge showed reduction in rLS/IBV.S vaccinated chickens, whereas oculo-nasal challenge did not. Abozeid *et al.* (1) developed rLS expressing the codon-optimized S glycoprotein of an Egyptian IBV variant strain. A high level of S protein expression was detected by both Western blot and immunofluorescence analyses. In accordance with other reports, their recombinant vaccine candidate was genetically stable, slightly attenuated, and showed replication patterns comparable to those of the parental LaSota virus. Single-dose vaccination in one-day-old SPF chickens with this vaccine provided significant protection against clinical disease after IBV challenge but did not reduce tracheal virus shedding.

We have successfully used the soluble trimeric IBV S-ectodomain protein of an Ark strain for immunization of chickens against virulent Ark IBV challenge (46). Therefore, while others have used the complete S gene as the transgene in the NDV LS vector, we report herein on development and protection conferred by a recombinant NDV LS expressing the S-ectodomain, that is, the S protein excluding the transmembrane anchor and short cytoplasmic domain of S2 that is not shown to the immune system during infection.

5.3. Materials and Methods

Chickens. White leghorn chickens were hatched from specific pathogen-free (SPF) embryonated eggs (Wayward Acres, Pickens, SC) and maintained in Horsfall-type isolators in biosafety level 2 facilities. Experimental procedures and animal care were performed in compliance with all

applicable federal and institutional animal care and use guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC International)-accredited institution.

Challenge viruses. Challenge was performed with Ark99 genotype virulent strains originating from different regions and showing limited genetic differences between them. In experiment 1, we used a virulent Ark-type virus (GenBank accession JN861120) previously characterized (54). In experiment 2 we challenged the birds with the previously described IBV Ark-type virulent strain AL/4614/98 (145) (GenBank accession #DQ458217; 98% nucleotide and 97% amino acid identity to Ark99 S1). The viruses were titered in SPF embryonated chicken eggs as accepted (59, 153) with slight modifications previously described (64). Vaccination and challenge in birds were performed with a total volume of 100 µl (25 µl in each nostril and each eye) of appropriately diluted virus stock.

Recombinant LaSota expressing IBV S-ectodomain (rLS/IBV.Se). Recombinant LaSota encoding the soluble trimeric Ark-type IBV S-ectodomain (S1 + S2 lacking the transmembrane domain and cytoplasmic tail) protein with a C-terminal artificial trimerization domain and Streptag II that we used for immunization of chickens and demonstrated remarkable protection against virulent Ark IBV challenge (46) was produced. It encodes amino acids 19-1097 of GenBank accession #ABY66333, with the amino acids of the furin cleavage site between S1 and S2 replaced with a protein linker. A gene encoding this protein including an N-terminal signal peptide was synthesized with codons optimized for chicken. The codon-optimized IBV S gene flanked by NDV LaSota gene start and gene end sequences was then inserted into the rLS vector between the phosphoprotein (P) and matrix (M) genes as an additional transcription unit using the In-Fusion®

PCR cloning kit (Clontech, Mountain View, CA) as described (147). Following confirmation of its sequence, the full-length cDNA infectious clone of pLS/IBV.S was used for rescue of the rLS/IBV.S virus. The virus was propagated in 10-day-old SPF chicken eggs and the nucleotide sequences of the rescued virus were determined by sequencing the RT-PCR products amplified from the viral genome as described previously (71). IBV S-ectodomain protein expression was confirmed by immunofluorescence using both an anti-Ark chicken serum and serum from chickens that had been immunized with recombinant S-ectodomain protein. The pathogenicity of rLS/IBV.S was evaluated by the intracerebral pathogenicity index (ICPI) as accepted (95) and by the mean death time (MDT) test in embryonated chicken eggs.

Experimental design. This work consisted of two experiments. In experiment 1, we evaluated protection conferred by single vaccination or prime and boost vaccination with rLS/IBV.Se against challenge using a virulent Ark strain. In experiment 2, we evaluated single vaccination with rLS/IBV.Se using an increased vaccination dose as well as a different Ark challenge strain. This experiment also included vaccination at different ages.

Experiment 1. Four treatment groups were established (n = 12 chickens/group). Chickens in groups 1 and 2 were vaccinated on day 1 of age with 10^4 EID_{50}/bird of rLS/IBV.Se. Group 2 was booster vaccinated with the same vaccine at the same dose on day 14 of age. Control groups 3, and 4 included unvaccinated/challenged and unvaccinated/non-challenged chickens, respectively. All chickens in groups 1 - 3 were challenged at 28 days of age with 10^5 EID_{50}/chicken of virulent IBV Ark (GenBank accession JN861120). Protection against challenge was evaluated 5 days after challenge by assessment of respiratory signs, tracheal histomorphometry, and viral load in tears. Absence or presence of respiratory rales (nasal and/or tracheal) were evaluated blindly by close

listening to each bird and incidence of chickens showing clinical signs compared by Fisher's exact test. Tracheal histomorphometry was performed as previously described (145). In brief, formalin-fixed sections of trachea were processed, embedded in paraffin, sectioned at 4-6 µm and stained with hematoxylin and eosin for histopathological examination. The tracheal mucosal thickness and the thickness of lymphocytic infiltration were measured using ImageJ (https://imagej.nih.gov/ij/download.html), and the average of five measurements for each chicken calculated. Data were analyzed by ANOVA. Relative viral loads in tears were determined by relative viral RNA levels measured by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR). IBV RNA to be used for qRT-PCR was extracted from tear samples using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA) following the manufacturer's protocol. Relative numbers of viral copies in RNA extracted from tear samples collected from individual chickens were determined by TaqMan© qRT-PCR as described (22). Viral RNA data were analyzed by one-way ANOVA followed by Tukey multiple comparisons post-test. Differences were considered significant with $P<0.05$.

Experiment 2. Five groups of chickens were established. Groups 1 and 2, consisting of 24 chickens each, were vaccinated at 1 and 10 days of age, respectively with 10^7 EID_{50}/chicken of rLS/IBV.Se. Groups 3 and 4 (n = 12-13 chickens/group) were age-matched unvaccinated chickens. Fourteen chickens of each groups 1 and 2, as well as control groups 3 and 4 were challenged at 20 days post-vaccination. Group 5 (n = 16) was the unvaccinated/not challenged control for both treatment groups, i.e. 8 chickens serve as unvaccinated/non-challenged controls for birds vaccinated on either day 1 or day 10, respectively. Challenge was performed with 10^4 EID_{50} /chicken of virulent Ark AL/4614/98. The remaining 10 vaccinated chickens of groups 1 and 2, which remained unchallenged, were maintained until 39 days post-vaccination to assess NDV and

IBV antibodies (described below). Protection was evaluated as described above by respiratory signs, viral load in tears, and tracheal histomorphometry.

Serum antibody. NDV antibody levels were determined both by ELISA and hemagglutination inhibition (HI) test. The commercially available NDV ELISA kit (Idexx Laboratories Inc., Westbrook, ME) was used following the manufacturer's recommendations. The HI test for NDV antibodies was performed as accepted (154). IBV Ark S1-specific antibody levels in sera were determined using a S1-specific ELISA as previously described (119) with minor modifications. Briefly, soluble trimeric recombinant Ark-type S1 protein (46) was produced in human embryonic kidney (HEK) 293T cells as described (158, 160) and used to coat ELISA plates (Nunc MaxiSorp, San Diego, CA). Each plate was incubated for 12 hrs at 4 C with 100 µl of phosphate-buffered saline (PBS) containing 0.2 µg/ml of recombinant S1 protein in each well. Wells were blocked for 3 h at 4 C with 200 µl/well of PBS containing 1% bovine serum albumin and 0.05% Tween 20. Chicken sera were diluted 1:100 in PBS and incubated in the wells for 30 min at room temperature. All following steps were performed with reagents of a commercial IBV ELISA kit (Idexx Laboratories Inc., Westbrook, ME) following the manufacturer's guidelines. Incidence of chickens showing IBV S1 antibodies (OD>3 SD above unvaccinated chickens) were compared by Fisher's test.

5.4. Results

IBV S-ectodomain protein expression from rLS/IBV.Se-infected DF-1 cells was confirmed by immunofluorescence assay (170) using both an anti-Ark chicken serum and serum from chickens that had been immunized with recombinant Ark S-ectodomain protein (Figure 5.1). The MDT and ICPI achieved by rLS/IBV.Se were>150 hrs and 0.0, respectively. Thus, the S gene

insert slightly reduced the pathogenicity of NDV LaSota, which had MTD of 110 hrs and ICPI of 0.15. Sequencing analysis of the RT-PCR products of the viral genome confirmed the sequence fidelity of rLS/IBV.Se (data not shown).

5.4.1. Experiment 1.

Protection by single or prime and booster vaccination. Single vaccination on day 1 of age did not confer measurable protection against challenge. Indeed, no differences in level of respiratory signs, viral load, or tracheal lesions were detected between vaccinated/challenged and unvaccinated /challenged chickens 5 days after challenge (data not shown). In contrast, the prime and boost vaccination regime with rLS/IBV.Se did confer some protection against challenge as seen in Figure 5.2. Individual blind listening for respiratory rales showed a significant difference (P = 0.04) in the incidence of chickens displaying respiratory signs between vaccinated and unvaccinated groups (Figure 5.2 A). Consistent with clinical sign findings, increased tracheal mucosal thickness (P<0.05) and slightly increased (not achieving statistical significance) lymphocyte infiltration were detected by histomorphometry in unvaccinated compared to vaccinated birds (Figure 5.2 B). In contrast, no difference was detected in IBV RNA levels in tears of vaccinated versus unvaccinated/challenged chickens (Figure 5.2 C).

5.4.2. Experiment 2.

Serum antibody. NDV and IBV S1 antibody levels detected in chickens vaccinated on day 1 or day 10 are shown in Figure 5.3. Vaccination with rLS/IBV.Se induced moderate NDV antibody responses with HI titers varying between 4 and 5 $\log_2$ (Figure 5.3 B). Slightly higher ELISA and HI antibody levels to NDV were observed in chickens vaccinated on day 10 versus day 1 vaccinated chickens but without differences achieving statistical significance (Figure 5.3 A and

B). Figure 5.3 C shows incidence of anti-IBVS1 antibodies in vaccinated chickens. A significantly greater number of chickens that were vaccinated on day 10 (10 of 10) showed presence of IBV S1 antibody compared to chicks vaccinated on day 1 (5 of 10) ($P = 0.03$).

Protection by single vaccination at increased age. The incidence of respiratory disease after challenge was greater in unvaccinated compared to chickens vaccinated at either 1 or 10 days of age (Figure 5.4). However, the difference between vaccinated and unvaccinated controls was significant only in chickens vaccinated on day 10 ($P = 0.005$). As was found in experiment 1, no differences in viral loads in lachrymal fluids were detected between vaccinated and unvaccinated chickens (Figure 5.4 B) irrespective of day of vaccination. The tracheal damage observed was consistent with the results of clinical signs. Both tracheal mucosal thickness and lymphocyte infiltration showed a tendency to be less in vaccinated/challenged chickens compared to unvaccinated/challenge chickens (Figure 5.5). However, differences between vaccinated and unvaccinated chickens were not statistically significant.

5.5. Discussion

Unlike other researchers who have used the complete S gene as the transgene in the NDV LS vector (1, 128), we expressed the S-ectodomain, that is, the S protein excluding the domain of S2 that is not shown to the immune system during infection. Expressing only the S-ectodomain, which cannot be inserted into the viral envelope in a recombinant viral vector, has the advantage over expression of the complete S protein, in that it avoids the bio-safety concern that the expression of the IBV S protein on the surface of NDV could have the potential to extend its tropism. We have previously shown that the trimeric S-ectodomain that we expressed efficiently binds tracheal epithelium and epithelium of other relevant chicken tissues, including choana, nasal

mucosa, lung, kidney, cecal tonsils, and cloaca, indicating that it is able to adopt a functional conformation, and thus present conformational neutralizing epitopes, without its transmembrane membrane (45). Furthermore, this recombinant S-ectodomain protein was able to induce a protective immune response when administered subcutaneously as a subunit vaccine with an adjuvant in a prime-boost regime (45). Expressing only the S-ectodomain, which cannot be inserted into the viral envelope in a recombinant viral vector, has the advantage over expression of the complete S protein, in that it avoids the bio-safety concern that the expression of the IBV S protein on the surface of NDV could have the potential to extend its tropism.

The results confirmed successful expression of S-ectodomain protein from rLS/IBV.Se. They also confirmed, in accordance with our previous experience (147) as well as experiences by others (128), that the S-ectodomain gene insert did not significantly alter the pathogenicity of the NDV LaSota vaccine strain but slightly reduced it making it more similar to an NDV B1-type vaccine strain, the mildest lentogenic NDV vaccine.

Shirvani *et al.* (128) vaccinated chickens at 1 day of age with rLS/IBV.S (without indicating the vaccine dose administered), and subsequently challenged with $10^{3.1}$ EID_{50} of an IBV M41 strain. Their results show limited protection against respiratory disease and absence of reduction of viral load in the tracheas of vaccinated/challenged chickens. Abozeid *et al.* (1) vaccinated 1-day-old chickens with 10^6 PFU/100µl of rLS expressing the S gene of an Egyptian IBV strain and challenged them with $10^{4.2}$ EID_{50}/bird of Egyptian virulent IBV strain. Their results were similar in that milder respiratory signs were detected in vaccinated chickens, but no reduction of viral load was apparent in the tracheas of challenged chickens. Compared to those studies, in the current first experiment, chickens were vaccinated with a lower dose (10^4 EID_{50}/bird) of

rLS/IBV.Se and challenged with a higher dose (10^5 EID_{50}/bird) of virulent IBV Ark. The vaccine and challenge doses used in the current study compared to the doses used by the above-mentioned scientists may explain why single immunization was completely ineffective against challenge in the current study.

Unlike the lack of effectiveness detected after single immunization, priming followed by booster immunization with rLS/IBV.Se was shown to confer some level of protection against challenge based on reduced incidence of signs and less severe tracheal lesions, although vaccination did not reduce the viral load in tears of challenged chickens. Prime+boost with rLS/IBV.S has been shown by others to provide protection against respiratory signs (1). A slight reduction of viral load in tracheas was also observed but unfortunately, histopathological evaluations were not included in those studies.

Early IBV vaccination, on the day of hatch, induces suboptimal IBV immune responses both in the systemic and mucosal compartments (119). Moreover, IBV vaccination at least 10 days after hatch induces more effective cross-protection than vaccination on day of hatch likely due to greater antibody affinity maturation (167). These results also find corroboration when vaccinating with rLS/IBV.S. Indeed, while little protection against challenge was detected in chickens vaccinated at 1-day-old, improved effectiveness was detected when chickens were vaccinated at 4 weeks of age (128). Therefore, we decided to compare vaccination on day 1 and day 10 of age with rLS/IBV Se. A higher vaccine dose was used in experiment 2, in an effort to compare results with those reported by others. Clearly, the use of a greater dose of recombinant vaccine virus (10^7 EID_{50}/chicken) proved to enhance the success of single vaccination substantially compared to experiment 1. However, such a high dose would be extremely difficult to achieve and use in commercial poultry operations; vaccine companies and regulating agencies tend to favor titers of

around 10^4 EID_{50} or less/bird. The comparison of protection after vaccination on day 1 of age versus day 10 of age corroborated previous findings (119). Specifically, respiratory signs were reduced more effectively in chickens vaccinated at 10 days of age. Similarly, NDV antibodies also seem to achieve slightly higher levels when vaccination was performed later. The incidence of antibodies to IBV measured using recombinant S1 protein-coated ELISA plates was significantly increased when vaccination was administered on day 10 compared to day 1. Interestingly, the viral loads in tear fluids of challenged chickens were not reduced by rLS/IBV.Se vaccination. Similar results, in which no reduction in viral load in the trachea became apparent from rLS/IBV.S vaccination, have been obtained by others (1, 128). Further work is needed to understand the immune responses induced by this recombinant virus that seems to provide some protection against the disease but does not reduce viral loads in the upper respiratory tract.

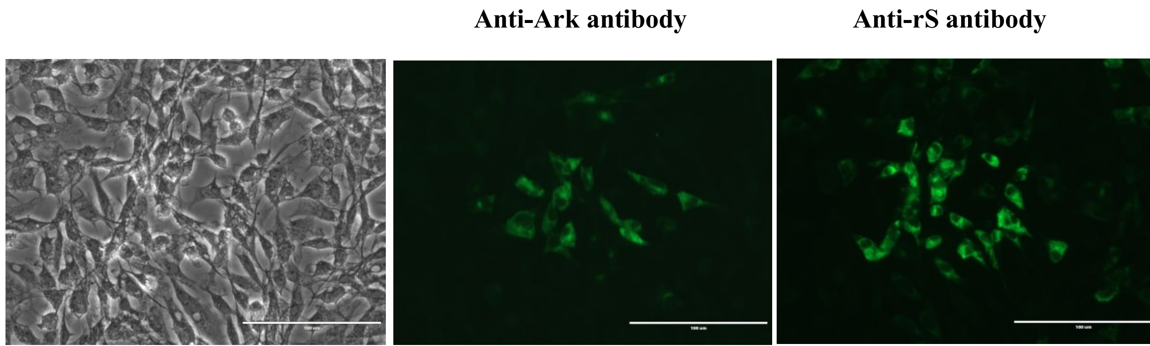

Figure 5.1. Detection of IBV S-ectodomain protein expression from recombinant NDV LaSota by immunofluorescence. Sera from chickens immunized with an Arkansas (Ark)-type strain and sera from chickens immunized with an Ark recombinant S protein were used to detect the S antigen in the cell culture.

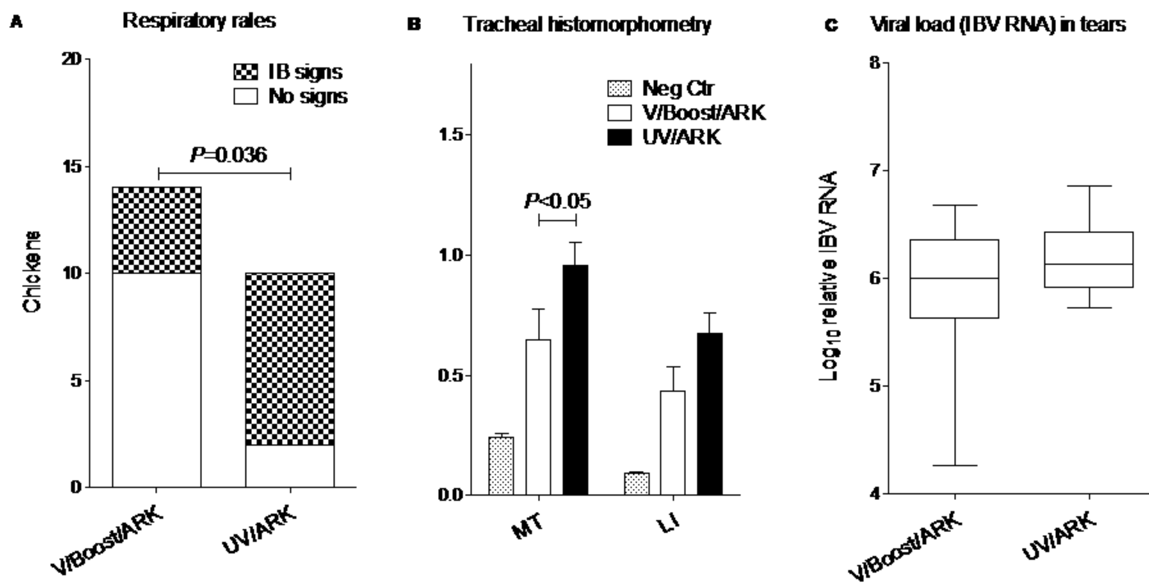

Figure 5.2. Protection conferred to chickens (n = 12/group) by prime at 1 day of age and booster at 14 days of age with 10^4 EID_{50}/bird of rLS/IBV.Se (Exp. 1) against Ark virulent challenge 28 days post-vaccination (V/Boost/ARK). UV/Ark = unvaccinated challenged controls. Signs, tracheal histomorphometry, and viral loads determined 5 days post-challenge. (A) Incidence of chickens with respiratory signs (boxes: 25th percentile, median, 75th percentile; whiskers:

minimum and maximum). Statistical difference by Fischer's test. (B) Tracheal histomorphometry including unvaccinated/non-challenged controls (Neg Ctr). MT = mucosal thickness. LI = Lymphocyte infiltration. Significant differences (*P*<0.05) by ANOVA. (C) Relative IBV RNA levels determined by quantitative RT-PCR in tears of individual chickens. Boxes and whiskers as above. Absence of significant differences determined by *Student t* test.

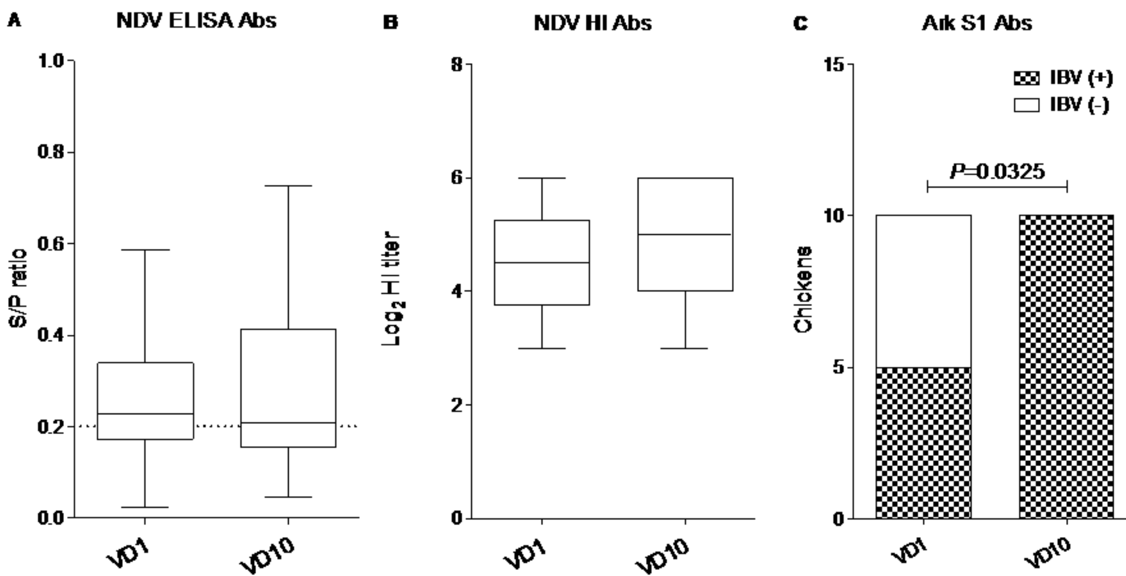

Figure 5.3. NDV serum antibodies and incidence of IBV S1 serum antibodies in chickens (n = 10/group) detected 39 days after single vaccination in chickens with 10^7 EID_{50}/chicken rLS/IBV.Se at 1 (VD1) or 10 (VD10) days of age (Exp. 2). NDV antibodies by (A) commercial ELISA (values over dotted line considered positive) or by (B) hemagglutination inhibition test. Data analyzed by *Student t* test. Ark S1 antibodies determined by ELISA using recombinant S1 protein coated plates. Incidence of chickens exhibiting IBV S1 antibodies analyzed by Fisher's test.

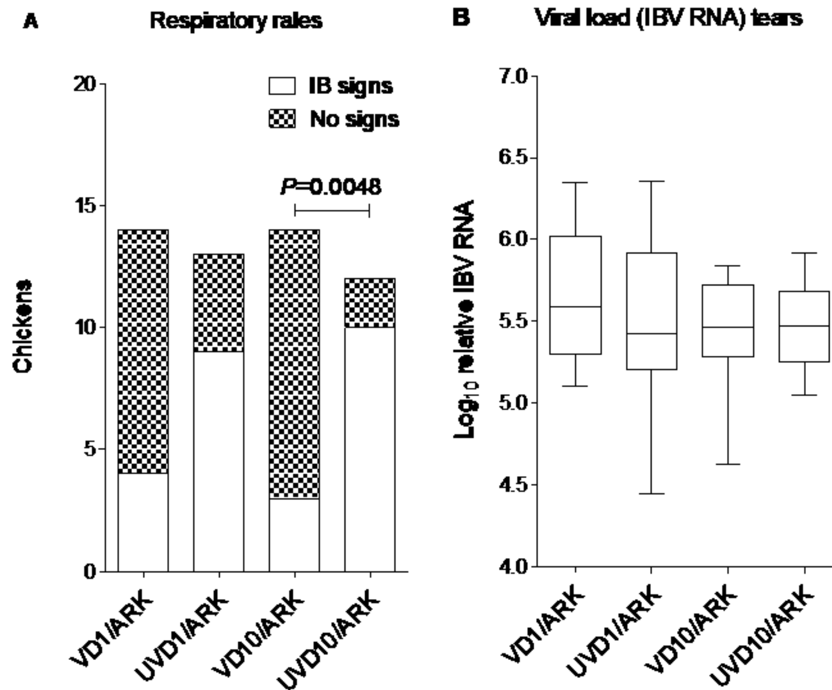

Figure 5.4. Protection conferred to chickens (n = 14/group) by single vaccination on day 1 (VD1) or 10 (VD10) of age with 10^7 EID_{50}/bird of rLS/IBV.Se (Exp. 2) against Ark virulent challenge (ARK) 20 days post-vaccination. UVD1 and UVD10 = age matched (day 1 and day 10) unvaccinated controls, respectively. (A) Signs, and (B) viral loads 5 days post-challenge determined and analyzed as described in Figure 5.2.

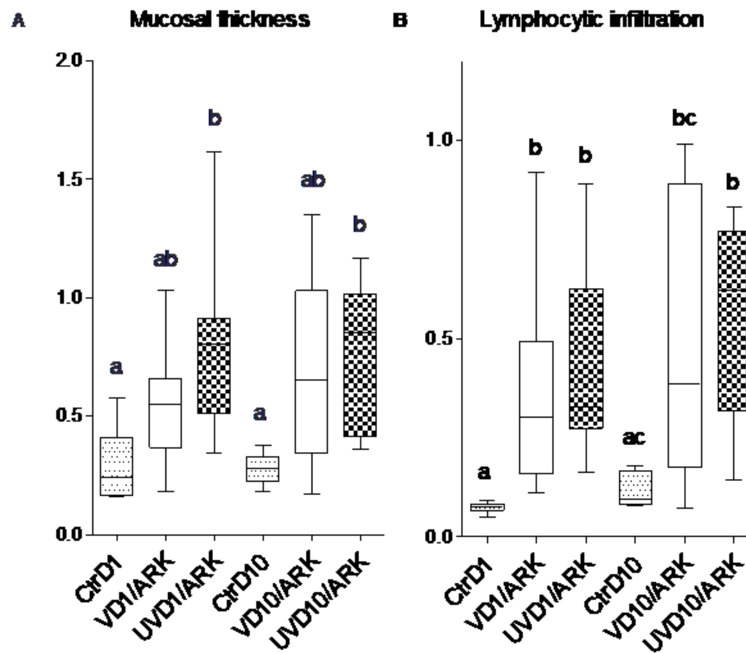

Figure 5.5. Tracheal histomorphometry 5 days post-challenge (ARK) in chickens (n = 14/group) singly vaccinated on day 1 (VD1) or 10 (VD10) of age with 10^7 EID_{50}/bird of rLS/IBV.Se (Exp. 2). UVD1 and UVD10 = age matched (day 1 and day 10) unvaccinated controls, respectively. CtrD1 and CtrD10 = unvaccinated/non-challenged age-matched controls. (A) Mucosal thickness and (B) lymphocyte infiltration (boxes: 25th percentile, median, 75th percentile; whiskers: minimum and maximum). Different letters indicate significant (*P*<0.05) by ANOVA.

CHAPTER 6.

Conclusions

The Arkansas-type IBV model provides evidence for immune selection in the vaccinated host that allows emergence of distinct viral subpopulations from virulent challenge virus.

Based on Arkansas-type IBV behavior in chickens, the population structure of live IBV vaccines influences innate immune responses, antibody avidity, and protection.

IBV vaccination on the day of hatch induces suboptimal IBV immune responses both in the systemic and mucosal compartments.

Postponing IBV vaccination beyond day one of age in chickens induces enhanced protection across IBV serotypes.

Vaccination with recombinant Newcastle disease virus expressing IBV S-ectodomain reduces clinical signs and lesions upon challenge but does not reduce viral load.

References

1. Abozeid, H. H., A. Paldurai, B. P. Varghese, S. K. Khattar, M. A. Afifi, S. Zouelfakkar, A. H. El-Deeb, M. F. El-Kady, and S. K. Samal. Development of a recombinant Newcastle disease virus-vectored vaccine for infectious bronchitis virus variant strains circulating in Egypt. Vet Res 50. 2019.

2. Albassam, M. A., R. W. Winterfield, and H. L. Thacker. Comparison of the nephropathogenicity of 4 strains of infectious bronchitis virus. Avian Dis 30:468-476. 1986.

3. Albini, B., G. Wick, E. Rose, and E. Orlans. Immunoglobulin rroduction in chicken Harderian glands. Int Arch Aller a Imm 47:23-34. 1974.

4. Alexander, D. J., and R. E. Gough. Isolation of avian infectious bronchitis virus from experimentally infected chickens. Res Vet Sci 23:344-347. 1977.

5. Ammayappan, A., C. Upadhyay, J. Gelb, Jr., and V. N. Vakharia. Identification of sequence changes responsible for the attenuation of avian infectious bronchitis virus strain Arkansas DPI. Arch Virol 154:495-499. 2009.

6. Ammayappan, A., C. Upadhyay, J. Gelb, and V. N. Vakharia. Complete genomic sequence analysis of infectious bronchitis virus Ark DPI strain and its evolution by recombination. Virol J 5:157. 2008.

7. Ariaans, M. P., P. M. V. de Haar, J. W. Lowenthal, W. van Eden, E. J. Hensen, and L. Vervelde. ELISPOT and intracellular cytokine staining: Novel assays for quantifying T cell responses in the chicken. Dev Comp Immunol 32:1398-1404. 2008.

8. Baba, T., K. Masumoto, S. Nishida, T. Kajikawa, and M. Mitsui. Harderian gland dependency of immunoglobulin A production in the lacrimal fluid of chicken. Immunology 65:67-71. 1988.

9. Banat, G. R., S. Tkalcic, J. A. Dzielawa, M. W. Jackwood, M. D. Saggese, L. Yates, R. Kopulos, W. E. Briles, and E. W. Collisson. Association of the chicken MHC B haplotypes with resistance to avian coronavirus. Dev Comp Immunol 39:430-437. 2013.

10. Bar-Shira, E., D. Sklan, and A. Friedman. Establishment of immune competence in the avian GALT during the immediate post-hatch period. Dev Comp Immunol 27:147-157. 2003.

11. Baric, R. S., S. A. Stohlman, and M. M. C. Lai. Characterization of replicative intermediate RNA of mouse hepatitis virus - presence of leader RNA sequences on nascent chains. J Virol 48:633-640. 1983.

12. Beach, J. R., and O. W. Schalm. A filterable virus, distinct from that of laryngotracheitis, the cause of a respiratory disease of chicks. Poultry Sci 15:199-206. 1936.

13. Beal, R. K., C. Powers, P. Wigley, P. A. Barrow, P. Kaiser, and A. L. Smith. A strong antigen-specific T-cell response is associated with age and genetically dependent resistance to avian enteric salmonellosis. Infect Immun 73:7509-7516. 2005.

14. Beaudette, F. R., and C. Hudson. Cultivation of the virus of infectious bronchitis. J Am Vet Med Assoc 90:51-60. 1937.

15. Belouzard, S., J. K. Millet, B. N. Licitra, and G. R. Whittaker. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses-Basel 4:1011-1033. 2012.

16. Bijlenga, G., J. K. A. Cook, J. Gelb, and J. J. de Wit. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. Avian Pathol 33:550-557. 2004.

17. Boltz, D. A., M. Nakai, and J. M. Bahr. Avian infectious bronchitis virus: A possible cause of reduced fertility in the rooster. Avian Dis 48:909-915. 2004.

18. Bosch, B. J., R. van der Zee, C. A. M. de Haan, and P. J. M. Rottier. The coronavirus spike protein Is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J Virol 77:8801-8811. 2003.

19. Boursnell, M. E. G., T. D. K. Brown, I. J. Foulds, P. F. Green, F. M. Tomley, and M. M. Binns. Completion of the sequence of the genome of the coronavirus avian infectious bronchitis virus. J Gen Virol 68:57-77. 1987.

20. Brierley, I., M. E. G. Boursnell, M. M. Binns, B. Bilimoria, V. C. Blok, T. D. K. Brown, and S. C. Inglis. An efficient ribosomal frame shifting signal in the polymerase encoding region of the coronavirus IBV. Embo J 6:3779-3785. 1987.

21. Brownlie, R., J. Z. Zhu, B. Allan, G. K. Mutwiri, L. A. Babiuk, A. Potter, and P. Griebel. Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. Mol Immunol 46:3163-3170. 2009.

22. Callison, S. A., D. A. Hilt, T. O. Boynton, B. F. Sample, R. Robison, D. E. Swayne, and M. W. Jackwood. Development and evaluation of a real-time Taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J Virol Methods 138:60-65. 2006.

23. Capua, I., R. Gough, M. Mancini, C. Casaccia, and C. Weiss. A 'novel' infectious bronchitis strain infecting broiler chickens in Italy. Zentralb Vet Riehe B 41:83-89. 1994.

24. Capua, I., Z. Minta, E. Karpinska, K. Mawditt, P. Britton, D. Cavanagh, and R. E. Gough. Co-circulation of four types of infectious bronchitis virus (793/B, 624/I, B1648 and Massachusetts). Avian Pathol 28:587-592. 1999.

25. Casais, R., B. Dove, D. Cavanagh, and P. Britton. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism. J Virol 77:9084-9089. 2003.

26. Cavanagh, D. Coronavirus avian infectious bronchitis virus. Vet Res 38:281-297. 2007.

27. Cavanagh, D., R. Casais, M. Armesto, T. Hodgson, S. Izadkhasti, D. Marc, F. Lin, I. Tarpey, and P. Britton. Manipulation of the infectious bronchitis coronavirus genome for vaccine development and analysis of the accessory proteins. Vaccine 25:5558-5562. 2007.

28. Cavanagh, D., P. J. Davis, J. K. A. Cook, D. Li, A. Kant, and G. Koch. Location of the amino acid differences in the S1 spike glycoprotein subunit of closely related serotypes of infectious bronchitis virus. Avian Pathol 21:33-43. 1992.

29. Cavanagh, D., P. J. Davis, J. H. Darbyshire, and R. W. Peters. Coronavirus IBV - virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus neutralizing or hemagglutination inhibiting antibody, or induce chicken tracheal protection. J Gen Virol 67:1435-1442. 1986.

30. Cavanagh, D., P. J. Davis, and A. P. A. Mockett. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res 11:141-150. 1988.

31. Chen, B., S. G. Fang, J. P. Tam, and D. X. Liu. Formation of stable homodimer via the C-terminal alpha-helical domain of coronavirus nonstructural protein 9 is critical for its function in viral replication. Virology 383:328-337. 2009.

32. Collisson, E. W., J. W. Pei, J. Dzielawa, and S. H. Seo. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. Dev Comp Immunol 24:187-200. 2000.

33. Cook, J. K. A. The classification of new serotypes of infectious bronchitis virus isolated from poultry flocks in Britain between 1981 and 1983. Avian Pathol 13:733-741. 1984.

34. Cook, J. K. A., and M. B. Huggins. Newly isolated serotypes of infectious bronchitis virus - their role in disease. Avian Pathol 15:129-&. 1986.

35. Corse, E., and C. E. Machamer. Infectious bronchitis virus E protein is targeted to the Golgi complex and directs release of virus-like particles. J Virol 74:4319-4326. 2000.

36. Crinion, R. A. P. Egg quality and production following infectious bronchitis virus exposure at one-day-old. Poultry Sci 51:582-585. 1972.

37. Crinion, R. A. P., and M. S. Hofstad. Pathogenicity of four serotypes of avian infectious bronchitis virus for the oviduct of young chickens of various ages. Avian Dis 16:351-363. 1972.

38. Darbyshire, J. H. Assessment of cross-immunity in chickens to strains of avian infectious bronchitis virus using tracheal organ cultures. Avian Pathol 9:179-184. 1980.

39. Darbyshire, J. H., J. K. Cook, and R. W. Peters. Organ culture studies on the efficiency of infection of chicken tissues with avian infectious bronchitis virus. Br J Exp Pathol 57:443-454. 1976.

40. Dauner, J. G., Y. J. Pan, A. Hildesheim, T. J. Kemp, C. Porras, and L. A. Pinto. Development and application of a GuHCl-modified ELISA to measure the avidity of anti-HPV L1 VLP antibodies in vaccinated individuals. Mol Cell Probe 26:73-80. 2012.

41. Davelaar, F. G., A. Noordzij, and J. A. Vanderdonk. A study on the synthesis and secretion of immunoglobulins by the Harderian gland of the fowl after eyedrop vaccination against infectious bronchitis at 1-day-old. Avian Pathol 11:63-79. 1982.

42. de Wit, J. J. Detection of infectious bronchitis virus. Avian Pathol 29:71-93. 2000.

43. de Wit, J. J., and J. K. A. Cook. Factors influencing the outcome of infectious bronchitis vaccination and challenge experiments. Avian Pathol 43:485-497. 2014.

44. De Wit, J. J., W. A. J. M. Swart, and T. H. F. Fabri. Efficacy of infectious bronchitis virus vaccinations in the field: association between the alpha-IBV IgM response, protection and vaccine application parameters. Avian Pathol 39:123-131. 2010.

45. Eldemery, F., K. S. Joiner, H. Toro, and V. L. van Santen. Protection against infectious bronchitis virus by spike ectodomain subunit vaccine. Vaccine 35:5864-5871. 2017.

46. Eldemery, F., Y. F. Li, Q. Z. Yu, V. L. van Santen, and H. Toro. Infectious bronchitis virus S2 of 4/91 expressed from recombinant virus does not protect against Ark-type challenge. Avian Dis 61:397-401. 2017.

47. Endomunoz, L. B., and J. T. Faragher. Avian infectious bronchitis - cross-protection studies using different Australian subtypes. Aust Vet J 66:345-348. 1989.

48. Estevez, C., D. King, B. Seal, and Q. Z. Yu. Evaluation of Newcastle disease virus chimeras expressing the Hemagglutinin-Neuraminidase protein of velogenic strains in the context of a mesogenic recombinant virus backbone. Virus Res 129:182-190. 2007.

49. Ewald, S. J., D. R. Kapczynski, E. J. Livant, D. L. Suarez, J. Ralph, S. McLeod, and C. Miller. Association of Mx1 Asn631 variant alleles with reductions in morbidity, early mortality, viral shedding, and cytokine responses in chickens infected with a highly pathogenic avian influenza virus. Immunogenetics 63:363-375. 2011.

50. Fabricant, J. The early history of infectious bronchitis. Avian Dis 42:648-650. 1998.

51. Fang, S. G., B. Chen, F. P. L. Tay, B. S. Ng, and D. X. Liu. An arginine-to-proline mutation in a domain with undefined functions within the helicase protein (Nsp13) is lethal to the coronavirus infectious bronchitis virus in cultured cells. Virology 358:136-147. 2007.

52. Farabaugh, P. J. Programmed translational frameshifting. Annu Rev Genet 30:507-528. 1996.

53. Fischer, F., C. F. Stegen, P. S. Masters, and W. A. Samsonoff. Analysis of constructed E gene mutants of mouse hepatitis virus confirms a pivotal role for E protein in coronavirus assembly. J Virol 72:7885-7894. 1998.

54. Gallardo, R. A., F. J. Hoerr, W. D. Berry, V. L. van Santen, and H. Toro. Infectious bronchitis virus in testicles and venereal transmission. Avian Dis 55:255-258. 2011.

55. Gallardo, R. A., V. L. van Santen, and H. Toro. Host intraspatial selection of infectious bronchitis virus populations. Avian Dis 54:807-813. 2010.

56. Geilhausen, H. E., F. B. Ligon, and P. D. Lukert. The pathogenesis of virulent and avirulent avian infectious bronchitis virus. Arch Ges Virusforsch 40:285-290. 1973.

57. Gelb, J., and S. S. Cloud. Effect of serial embryo passage of an Arkansas-type avian infectious-bronchitis virus isolate on clinical response, virus recovery, and immunity. Avian Dis 27:679-687. 1983.

58. Gelb, J., Jr., and M. W. Jackwood. Infectious bronchitis. In: A laboratory manual for the isolation, identification and characterization of avian pathogens. L. Dufour-Zavala, D. E. Swayne, J. R. Glisson, J. E. Pearson, W. M. Reed, M. W. Jackwood and P. R. Woolcock, eds. Am Assoc Avian Pathol, Athens, GA. pp 146-149. 2008.

59. Gelb, J., Jr., and M. W. Jackwood. Infectious bronchitis. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 6th ed. L. Dufour-Zavala, M. W. Jackwood, M. D. Lee, B. Lupiani, W. M. Reed, E. Spackman and P. R. Woolcock, eds. Am Assoc Avian Pathol. pp 233-238. 2016.

60. Gelb, J., and S. L. Killian. Serum antibody response of chickens to multiple inoculations with infectious bronchitis virus serotypes. J Am Vet Med Assoc 185:337-337. 1984.

61. Gelb, J., W. A. Nix, and S. D. Gellman. Infectious bronchitis virus antibodies in tears and their relationship to immunity. Avian Dis 42:364-374. 1998.

62. Gelb, J., B. E. Perkins, J. K. Rosenberger, and P. H. Allen. Serologic and cross protection studies with several infectious bronchitis virus isolates from Delmarva reared broiler chickens. Avian Dis 25:655-666. 1981.

63. Ghetas, A. M., G. E. Thaxton, C. Breedlove, V. L. van Santen, and H. Toro. Effects of adaptation of infectious bronchitis virus Arkansas attenuated vaccine to embryonic kidney cells. Avian Dis 59:106-113. 2015.

64. Ghetas, A. M., V. L. van Santen, K. Joiner, and H. Toro. Kidney cell-adapted infectious bronchitis virus Arkansas Delmarva poultry industry vaccine confers effective protection against challenge. Avian Dis 60:418-423. 2016.

65. Glahn, R. P., R. F. Wideman, and B. S. Cowen. Order of exposure to high dietary calcium and Gray strain infectious-bronchitis virus alters renal function and the incidence of urolithiasis. Poultry Sci 68:1193-1204. 1989.

66. Guo, X. H., A. J. M. Rosa, D. G. Chen, and X. Q. Wang. Molecular mechanisms of primary and secondary mucosal immunity using avian infectious bronchitis virus as a model system. Vet Immunol Immunop 121:332-343. 2008.

67. Gurjar, R. S., S. L. Gulley, and F. W. van Ginkel. Cell-mediated immune responses in the head-associated lymphoid tissues induced to a live attenuated avian coronavirus vaccine. Dev Comp Immunol 41:715-722. 2013.

68. Hewson, K. A., J. Ignjatovic, G. F. Browning, J. M. Devlin, and A. H. Noormohammadi. Infectious bronchitis viruses with naturally occurring genomic rearrangement and gene deletion. Arch Virol 156:245-252. 2011.

69. Hofstad, M. S. Cross-immunity in chickens using seven isolates of avian infectious bronchitis virus. Avian Dis 25:650-654. 1981.

70. Hofstad, M. S., and H. W. Yoder. Avian infectious bronchitis - virus distribution in tissues of chicks. Avian Dis 10:230-239. 1966.

71. Hu, H. X., J. P. Roth, C. N. Estevez, L. Zsak, B. Liu, and Q. Z. Yu. Generation and evaluation of a recombinant Newcastle disease virus expressing the glycoprotein (G) of avian metapneumovirus subgroup C as a bivalent vaccine in turkeys. Vaccine 29:8624-8633. 2011.

72. Ignjatovic, J., D. F. Ashton, R. Reece, P. Scott, and P. Hooper. Pathogenicity of Australian strains of avian infectious bronchitis virus. J Comp Pathol 126:115-123. 2002.

73. Jackwood, M. W., and S. de Wit. Infectious Bronchitis. In: Diseases of Poultry, 13th ed. D. E. Swayne, J. R. Glisson, L. R. McDougald, L. K. Nolan, D. L. Suarez and V. Nair, eds. Blackwell Publishing, Ames, Iowa, U. S. pp 139-160. 2013.

74. Jackwood, M. W., D. A. Hilt, C. W. Lee, H. M. Kwon, S. A. Callison, K. M. Moore, H. Moscoso, H. Sellers, and S. Thayer. Data from 11 years of molecular typing infectious bronchitis virus field isolates. Avian Dis 49:614-618. 2005.

75. Jackwood, M. W., D. A. Hilt, A. W. McCall, C. N. Polizzi, E. T. McKinley, and S. M. Williams. Infectious bronchitis virus field vaccination coverage and persistence of Arkansas-type viruses in commercial broilers. Avian Dis 53:175-183. 2009.

76. Jackwood, M. W., and D. H. Lee. Different evolutionary trajectories of vaccine-controlled and non-controlled avian infectious bronchitis viruses in commercial poultry. Plos One 12. 2017.

77. Jeurissen, S. H. M., E. M. Janse, G. Koch, and G. F. Deboer. Postnatal development of mucosa-associated lymphoid-tissues in chickens. Cell Tissue Res 258:119-124. 1989.

78. Johnson, M. A., C. Pooley, J. Ignjatovic, and S. G. Tyack. A recombinant fowl adenovirus expressing the S1 gene of infectious bronchitis virus protects against challenge with infectious bronchitis virus. Vaccine 21:2730-2736. 2003.

79. Jones, C. L., R. A. Macdonald, C. S. Hosking, and D. M. Roberton. Estimating the relative avidity of mucosal IgA for antigen. J Immunol Methods 105:111-117. 1987.

80. Jungherr, E. L., T. W. Chomiak, and R. E. Luginbuhl. Immunologic differences in strains of infectious bronchitis virus. Proc 60th Ann Meet U.S. live Stk sanit Assoc, Chicago, Illinois, USA. pp 203-209. 1956.

81. Juul-Madsen, H. R., M. Munch, K. J. Handberg, P. Sorensen, A. A. Johnson, L. R. Norup, and P. H. Jorgensen. Serum levels of mannan-binding lectin in chickens prior to and during experimental infection with avian infectious bronchitis virus. Poultry Sci 82:235-241. 2003.

82. Juul-Madsen, H. R., L. R. Norup, K. J. Handberg, and P. H. Jordensen. Mannan-binding lectin (MBL) serum concentration in relation to propagation of infectious bronchitis virus (IBV) in chickens. Viral Immunol 20:562-570. 2007.

83. Kant, A., G. Koch, D. J. Vanroozelaar, J. G. Kusters, F. A. J. Poelwijk, and B. A. M. Vanderzeijst. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J Gen Virol 73:591-596. 1992.

84. Kemper, C., and J. P. Atkinson. T-cell regulation: with complements from innate immunity. Nat Rev Immunol 7:9-18. 2007.

85. King, A. M. Q., E. J. Lefkowitz, A. R. Mushegian, M. J. Adams, B. E. Dutilh, A. E. Gorbalenya, B. Harrach, R. L. Harrison, S. Junglen, N. J. Knowles, A. M. Kropinski, M. Krupovic, J. H. Kuhn, M. L. Nibert, L. Rubino, S. Sabanadzovic, H. Sanfacon, S. G. Siddell, P. Simmonds, A. Varsani, F. M. Zerbini, and A. J. Davison. Changes to taxonomy and the International Code of Virus Classification and Nomenclature ratified by the International Committee on Taxonomy of Viruses (2018). Arch Virol 163:2601-2631. 2018.

86. Kint, J., A. Dickhout, J. Kutter, H. J. Maier, P. Britton, J. Koumans, G. P. Pijlman, J. J. Fros, G. F. Wiegertjes, and M. Forlenza. Infectious bronchitis coronavirus inhibits STAT1 signaling and requires accessory proteins for resistance to type I interferon activity. J Virol 89:12047-12057. 2015.

87. Kint, J., M. A. Langereis, H. J. Maier, P. Britton, F. J. van Kuppeveld, J. Koumans, G. F. Wiegertjes, and M. Forlenza. Infectious bronchitis coronavirus limits interferon production by inducing a host shutoff that requires accessory protein 5b. J Virol 90:7519-7528. 2016.

88. Kjaerup, R. M., T. S. Dalgaard, L. R. Norup, I. M. Bergman, P. Sorensen, and H. R. Juul-Madsen. Adjuvant effects of mannose-binding lectin ligands on the immune response to infectious

bronchitis vaccine in chickens with high or low serum mannose-binding lectin concentrations. Immunobiology 219:263-274. 2014.

89. Klieve, A. V., and R. B. Cumming. Respiratory disease and immunity to challenge produced by Australian strains of infectious-bronchitis virus. Avian Pathol 19:305-312. 1990.

90. Knoops, K., M. Kikkert, S. H. E. van den Worm, J. C. Zevenhoven-Dobbe, Y. van der Meer, A. J. Koster, A. M. Mommaas, and E. J. Snijder. SARS-coronavirus replication is supported by a reticulovesicular network of modified endoplasmic reticulum. Plos Biol 6:1957-1974. 2008.

91. Kwon, H. M., M. W. Jackwood, and J. Gelb. Differentiation of infectious bronchitis virus serotypes using polymerase chain reaction and restriction fragment length polymorphism analysis. Avian Dis 37:194-202. 1993.

92. Lambrechts, C., M. Pensaert, and R. Ducatelle. Challenge experiments to evaluate cross-protection induced at the trachea and kidney level by vaccine strains and Belgian nephropathogenic isolates of avian infectious bronchitis virus. Avian Pathol 22:577-590. 1993.

93. Li, F. Structure, function, and evolution of coronavirus spike proteins. Annu Rev Virol 3:237-261. 2016.

94. Lim, T. H., M. S. Kim, J. H. Jang, D. H. Lee, J. K. Park, H. N. Youn, J. B. Lee, S. Y. Park, I. S. Choi, and C. S. Song. Live attenuated nephropathogenic infectious bronchitis virus vaccine provides broad cross protection against new variant strains. Poultry Sci 91:89-94. 2012.

95. Loendt, B. Z., and D. A. Alexander. Newcastle disease virus and other avian paramyxoviruses. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 6th ed. S. M. Williams, ed. Am Assoc Avian Pathol, Athens, GA. pp 259-267. 2016.

96. Lohr, J. E. The occurrence of serum neutralizing antibodies against several variant strains of the infectious bronchitis virus in South Wurttemberg. Berl Munch Tierarztl 99:411-414. 1986.

97. Lowenthal, J. W., T. E. Connick, P. G. Mcwaters, and J. J. York. Development of T-cell immune responsiveness in the chicken. Immunol Cell Biol 72:115-122. 1994.

98. Lucio, B., and J. Fabricant. Tissue tropism of three cloacal isolates and Massachusetts strain of infectious bronchitis virus. Avian Dis 34:865-870. 1990.

99. Ma, Y. Y., L. J. Wu, N. Shaw, Y. Gao, J. Wang, Y. N. Sun, Z. Y. Lou, L. M. Yan, R. G. Zhang, and Z. H. Rao. Structural basis and functional analysis of the SARS coronavirus nsp14-nsp10 complex. P Natl Acad Sci USA 112:9436-9441. 2015.

100. Maslak, D. M., and D. L. Reynolds. B cells and T-lymphocyte subsets of the head-associated lymphoid tissues of the chicken. Avian Dis 39:736-742. 1995.

101. Mast, J., and B. M. Goddeeris. Development of immunocompetence of broiler chickens. Vet Immunol Immunop 70:245-256. 1999.

102. Masters, P. S., and S. Perlman. Coronaviridae. In: Fields Virology, 6th ed. D. M. Knipe and P. M. Howley, eds. Lippincott Williams and Wilkins, Philadelphia, USA. pp 825-858. 2013.

103. Matthijs, M. G. R., J. H. H. van Eck, J. J. de Wit, A. Bouma, and J. A. Stegeman. Effect of IBV-H120 vaccination in broilers on colibacillosis susceptibility after infection with a virulent Massachusetts-type IBV strain. Avian Dis 49:540-545. 2005.

104. McKinley, E. T., D. A. Hilt, and M. W. Jackwood. Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. Vaccine 26:1274-1284. 2008.

105. Michailidis, G., A. Theodoridis, and M. Avdi. Transcriptional profiling of Toll-like receptors in chicken embryos and in the ovary during sexual maturation and in response to Salmonella enteritidis infection. Anim Reprod Sci 122:294-302. 2010.

106. Mockett, A. P. A., J. K. A. Cook, and M. B. Huggins. Maternally-derived antibody to infectious bronchitis virus - its detection in chick trachea and serum and its role in protection. Avian Pathol 16:407-416. 1987.

107. Mondal, S. P., and S. A. Naqi. Maternal antibody to infectious bronchitis virus: its role in protection against infection and development of active immunity to vaccine. Vet Immunol Immunop 79:31-40. 2001.

108. Moore, K. M., M. W. Jackwood, and D. A. Hilt. Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. Arch Virol 142:2249-2256. 1997.

109. Nakamura, K., J. K. A. Cook, K. Otsuki, M. B. Huggins, and J. A. Frazier. Comparative study of respiratory lesions in 2 chicken lines of different susceptibility infected with infectious bronchitis virus - histology, ultrastructure and immunohistochemistry. Avian Pathol 20:241-257. 1991.

110. Namy, O., S. J. Moran, D. I. Stuart, R. J. C. Gilbert, and I. Brierley. A mechanical explanation of RNA pseudoknot function in programmed ribosomal frameshifting. Nature 441:244-247. 2006.

111. Ndegwa, E. N., K. S. Joiner, H. Toro, F. W. van Ginkel, and V. L. van Santen. The proportion of specific viral subpopulations in attenuated ArkDPI infectious bronchitis vaccines influences vaccination outcome. Avian Dis 56:642-653. 2012.

112. Ndegwa, E. N., H. Toro, and V. L. van Santen. Comparison of vaccine subpopulation selection, viral loads, vaccine virus persistence in trachea and cloaca, and mucosal antibody responses after vaccination with two different Arkansas Delmarva Poultry Industry -derived infectious bronchitis virus vaccines. Avian Dis 58:102-110. 2014.

113. Niedorf, H. R., and B. Wolters. Development of Harderian gland in chicken - light and electron microscopic investigations. Invest Cell Pathol 1:205-215. 1978.

114. Nix, W. A., D. S. Troeber, B. F. Kingham, C. L. Keeler, and J. Gelb. Emergence of subtype strains of the Arkansas serotype of infectious bronchitis virus in Delmarva broiler chickens. Avian Dis 44:568-581. 2000.

115. Owen, R. L., B. S. Cowen, A. L. Hattel, S. A. Naqi, and R. A. Wilson. Detection of viral antigen following exposure of one-day-old chickens to the Holland 52 strain of infectious bronchitis virus. Avian Pathol 20:663-673. 1991.

116. Parsons, D., M. M. Ellis, D. Cavanagh, and J. K. A. Cook. Characterization of an infectious bronchitis virus isolated from vaccinated broiler breeder flocks. Vet Rec 131:408-411. 1992.

117. Pasternak, A. O., W. J. M. Spaan, and E. J. Snijder. Nidovirus transcription: how to make sense ... ? J Gen Virol 87:1403-1421. 2006.

118. Reemers, S. S., D. van Leenen, M. J. G. Koerkamp, D. van Haarlem, P. van de Haar, W. van Eden, and L. Vervelde. Early host responses to avian influenza A virus are prolonged and enhanced at transcriptional level depending on maturation of the immune system. Mol Immunol 47:1675-1685. 2010.

119. Saiada, F., F. Eldemery, R. A. Zegpi, S. L. Gulley, A. Mishra, V. L. van Santen, and H. Toro. Early vaccination of chickens induces suboptimal immunity against infectious bronchitis virus. Avian Dis 63:38-47. 2019.

120. Sawicki, S. G., D. L. Sawicki, and S. G. Siddell. A contemporary view of coronavirus transcription. J Virol 81:20-29. 2007.

121. Schalk, A. F., and M. C. Hawn. An apparently new respiratory disease of baby chicks. J Am Vet Med Assoc 78:413-423. 1931.

122. Schelle, B., N. Karl, B. Ludewig, S. G. Siddell, and V. Thiel. Selective replication of coronavirus genomes that express nucleocapsid protein. J Virol 79:6620-6630. 2005.

123. Scott, T. R., M. L. Savage, and I. Olah. Plasma cells of the chicken Harderian gland. Poultry Sci 72:1273-1279. 1993.

124. Seo, S. H., and E. W. Collisson. Specific cytotoxic T lymphocytes are involved in in vivo clearance of infectious bronchitis virus. J Virol 71:5173-5177. 1997.

125. Seo, S. H., J. W. Pei, W. E. Briles, J. Dzielawa, and E. W. Collisson. Adoptive transfer of infectious bronchitis virus primed alpha beta T cells bearing CD8 antigen protects chicks from acute infection. Virology 269:183-189. 2000.

126. Sevoian, M., and P. P. Levine. Effects of infectious bronchitis on the reproductive tracts, egg production, and egg quality of laying chickens. Avian Dis 1:136-164. 1957.

127. Shang, J., Y. Zheng, Y. Yang, C. Liu, Q. Geng, C. Luo, W. Zhang, and F. Li. Cryo-EM structure of infectious bronchitis coronavirus spike protein reveals structural and functional evolution of coronavirus spike proteins. Plos Pathog 14:e1007009. 2018.

128. Shirvani, E., A. Paldurai, V. K. Manoharan, B. P. Varghese, and S. K. Samal. A recombinant Newcastle disease virus (NDV) expressing S protein of infectious bronchitis virus (IBV) protects chickens against IBV and NDV. Sci Rep-Uk 8. 2018.

129. Siller, W. G., and R. B. Cumming. The histopathology of an interstitial nephritis in the fowl produced experimentally with infectious bronchitis virus. J Pathol 114:163-173. 1974.

130. Singh, S., H. Toro, D. C. Tang, W. E. Briles, L. M. Yates, R. T. Kopulos, and E. W. Collisson. Non-replicating adenovirus vectors expressing avian influenza virus hemagglutinin and nucleocapsid proteins induce chicken specific effector, memory and effector memory CD8(+) T lymphocytes. Virology 405:62-69. 2010.

131. Smith, E. C., and M. R. Denison. Coronaviruses as DNA wannabes: a new model for the regulation of RNA virus replication fidelity. Plos Pathog 9. 2013.

132. Snijder, E. J., P. J. Bredenbeek, J. C. Dobbe, V. Thiel, J. Ziebuhr, L. L. M. Poon, Y. Guan, M. Rozanov, W. J. M. Spaan, and A. E. Gorbalenya. Unique and conserved features of genome and proteome of SARS-coronavirus, an early split-off from the coronavirus group 2 lineage. J Mol Biol 331:991-1004. 2003.

133. Spaan, W., D. Cavanagh, and M. C. Horzinek. Coronaviruses - structure and genome expression. J Gen Virol 69:2939-2952. 1988.

134. Stadler, K., V. Masignani, M. Eickmann, S. Becker, S. Abrignani, H. D. Klenk, and R. Rappuoli. SARS - beginning to understand a new virus. Nat Rev Microbiol 1:209-218. 2003.

135. Stern, D. F., and B. M. Sefton. Coronavirus proteins - biogenesis of avian infectious bronchitis virus virion proteins. J Virol 44:794-803. 1982.

136. Tan, Y. W., T. S. Fung, H. Y. Shen, M. Huang, and D. X. Liu. Coronavirus infectious bronchitis virus non-structural proteins 8 and 12 form stable complex independent of the non-translated regions of viral RNA and other viral proteins. Virology 513:75-84. 2018.

137. Thor, S. W., D. A. Hilt, J. C. Kissinger, A. H. Paterson, and M. W. Jackwood. Recombination in avian gamma-coronavirus infectious bronchitis virus. Viruses-Basel 3:1777-1799. 2011.

138. Tibbles, K. W., I. Brierley, D. Cavanagh, and T. D. K. Brown. Characterization in vitro of an autocatalytic processing activity associated with the predicted 3C-like proteinase domain of the coronavirus avian infectious bronchitis virus. J Virol 70:1923-1930. 1996.

139. Toro, H., and I. Fernandez. Avian infectious bronchitis - specific lacrimal IgA level and resistance against challenge. J Vet Med B 41:467-472. 1994.

140. Toro, H., P. Lavaud, P. Vallejos, and A. Ferreira. Transfer of IgG from serum to lacrimal fluid in chickens. Avian Dis 37:60-66. 1993.

141. Toro, H., D. L. Suarez, D. C. C. Tang, F. W. van Ginkel, and C. Breedlove. Avian influenza mucosal vaccination in chickens with replication-defective recombinant adenovirus vaccine. Avian Dis 55:43-47. 2011.

142. Toro, H., D. C. C. Tang, D. L. Suarez, J. F. Zhang, and Z. K. Shi. Protection of chickens against avian influenza with non-replicating adenovirus-vectored vaccine. Vaccine 26:2640-2646. 2008.

143. Toro, H., V. L. van Santen, A. M. Ghetas, and K. S. Joiner. Cross-protection by infectious bronchitis viruses under controlled experimental conditions. Avian Dis 59:532-536. 2015.

144. Toro, H., V. L. van Santen, and M. W. Jackwood. Genetic diversity and selection regulates evolution of infectious bronchitis virus. Avian Dis 56:449-455. 2012.

145. Toro, H., V. L. van Santen, L. Li, S. B. Lockaby, E. van Santen, and F. J. Hoerr. Epidemiological and experimental evidence for immunodeficiency affecting avian infectious bronchitis. Avian Pathol 35:455-464. 2006.

146. Toro, H., J. F. Zhang, R. A. Gallardo, V. L. van Santen, F. W. van Ginkel, K. S. Joiner, and C. Breedlove. S1 of distinct ibv population expressed from recombinant adenovirus confers protection against challenge. Avian Dis 58:211-215. 2014.

147. Toro, H., W. Zhao, C. Breedlove, Z. Y. Zhang, V. van Santen, and Q. Z. Yu. Infectious bronchitis virus S2 expressed from recombinant virus confers broad protection against challenge. Avian Dis 58:83-89. 2014.

148. van Ginkel, F. W., J. Padgett, G. Martinez-Romero, M. S. Miller, K. S. Joiner, and S. L. Gulley. Age-dependent immune responses and immune protection after avian coronavirus vaccination. Vaccine 33:2655-2661. 2015.

149. van Ginkel, F. W., V. L. van Santen, S. L. Gulley, and H. Toro. Infectious bronchitis virus in the chicken Harderian gland and lachrymal fluid: viral load, infectivity, immune cell responses, and effects of viral immunodeficiency. Avian Dis 52:608-617. 2008.

150. van Santen, V. L., S. Farjana, K. S. Joiner, and H. Toro. Embryonic kidney cell-adapted infectious bronchitis virus spike protein shows a reduced host cell binding spectrum. In: Proceedings of the 9th International Symposium on Avian Corona- and Pneumoviruses and Complicating Pathogens. H. Verheije, S. de Wit, S. van Beurden, A. Grone and E. Weerts, eds., Ovimex, Leusden, The Netherlands. pp 111–114. 2016.

151. van Santen, V. L., and H. Toro. Rapid selection in chickens of subpopulations within ArkDPI-derived infectious bronchitis virus vaccines. Avian Pathol 37:293-306. 2008.

152. Villegas, P. Titration of biological suspensions. In: A laboratory manual for the isolation, identification and characterization of avian pathogens. L. Dufour-Zavala, D. E. Swayne, J. R. Glisson, J. E. Pearson, W. M. Reed, M. W. Jackwood and P. R. Woolcock, eds. Am Assoc Avian Pathol, Athens, GA. pp 217-221. 2008.

153. Villegas, P. Titration of biological suspensions. In: A laboratory manual for the isolation, identification and characterization of avian pathogens, 6th ed. L. Dufour-Zavala, M. W. Jackwood, M. D. Lee, B. Lupiani, W. M. Reed, E. Spackman and P. R. Woolcock, eds. Am Assoc Avian Pathol, Athens, GA. pp 355-360. 2016.

154. Wackenell, P. S., S. G. Thayer, and C. W. Beard. Serologic procedures. In: A laboratory manual for the isolation and identification, and characterization of avian pathogens, 6th ed. S. M. Williams, ed. Am Assoc Avian Pathol, Athens, GA. pp 343-354. 2016.

155. Wang, X. Q., A. J. M. Rosa, H. N. Oliverira, G. J. M. Rosa, X. S. Guo, M. Travnicek, and T. Girshick. Transcriptome of local innate and adaptive immunity during early phase of infectious bronchitis viral infection. Viral Immunol 19:768-774. 2006.

156. Watabe, M., and B. Glick. Graft versus host response as influenced by the origin of the cell, age of chicken, and cellular interactions. Poultry Sci 62:1317-1324. 1983.

157. Wells, L. L., V. K. Lowry, J. R. DeLoach, and M. H. Kogut. Age-dependent phagocytosis and bactericidal activities of the chicken heterophil. Dev Comp Immunol 22:103-109. 1998.

158. Wickramasinghe, I. N., R. P. de Vries, A. Grone, C. A. de Haan, and M. H. Verheije. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. J Virol 85:8903-8912. 2011.

159. Wickramasinghe, I. N. A., R. P. de Vries, E. A. W. S. Weerts, S. J. van Beurden, W. Peng, R. McBride, M. Ducatez, J. Guy, P. Brown, N. Eterradossi, A. Grone, J. C. Paulson, and M. H. Verheijea. Novel receptor specificity of avian gammacoronaviruses that cause enteritis. J Virol 89:8783-8792. 2015.

160. Wickramasinghe, I. N. A., and M. H. Verheije. Protein histochemistry using coronaviral spike proteins: studying binding profiles and sialic acid requirements for attachment to tissues. Methods Mol Biol 1282:155-163. 2015.

161. Winterfield, R. W., A. M. Fadly, and F. J. Hoerr. Vaccination and revaccination with a holland (H) strain of infectious bronchitis virus. Avian Dis 20:369-374. 1976.

162. Yin, C. G., C. S. Zhang, A. M. Zhang, H. W. Qin, X. Q. Wang, L. X. Du, and G. P. Zhao. Expression analyses and antiviral properties of the Beijing-You and White Leghorn myxovirus resistance gene with different amino acids at position 631. Poultry Sci 89:2259-2264. 2010.

163. Yu, L., Y. H. Jiang, S. Low, Z. L. Wang, S. J. Nam, W. Liu, and J. Kwang. Characterization of three infectious bronchitis virus isolates from China associated with proventriculus in vaccinated chickens. Avian Dis 45:416-424. 2001.

164. Yu, Q., J. P. Roth, H. Hu, C. N. Estevez, W. Zhao, and L. Zsak. Protection by recombinant Newcastle disease viruses (NDV) expressing the glycoprotein (G) of avian metapneumovirus (aMPV) subtype A or B against challenge with virulent NDV and aMPV. World J Vaccine 3:130-139. 2013.

165. Yuan, Y., Z. P. Zhang, Y. N. He, W. S. Fan, Z. H. Dong, L. H. Zhang, X. K. Sun, L. L. Song, T. C. Wei, M. L. Mo, and P. Wei. Protection against virulent infectious bronchitis virus challenge conferred by a recombinant baculovirus co-expressing S1 and N proteins. Viruses-Basel 10:347. 2018.

166. Zegpi, R. A., C. Breedlove, V. L. van Santen, C. R. Rasmussen-Ivey, and H. Toro. Kidney cell-adapted infectious bronchitis ArkDPI vaccine is stable and protective. Avian Dis 61:221-228. 2017.

167. Zegpi, R. A., S. L. Gulley, V. L. van Santen, K. S. Joiner, and H. Toro. Infectious bronchitis virus vaccination at day 1 of age further limits cross protection. Avian Dis 63:302-309. 2019.

168. Zeshan, B., L. L. Zhang, J. Bai, X. L. Wang, J. R. Xu, and P. Jiang. Immunogenicity and protective efficacy of a replication-defective infectious bronchitis virus vaccine using an adenovirus vector and administered in ovo. J Virol Methods 166:54-59. 2010.

169. Zhang, D. C., Y. Q. Long, M. Li, J. F. Gong, X. H. Li, J. Lin, J. L. Meng, K. K. Gao, R. L. Zhao, and T. M. Jin. Development and evaluation of novel recombinant adenovirus-based vaccine candidates for infectious bronchitis virus and Mycoplasma gallisepticum in chickens. Avian Pathol 47:213-222. 2018.

170. Zhao, W., S. Spatz, Z. Y. Zhang, G. Y. Wen, M. Garcia, L. Zsak, and Q. Z. Yu. Newcastle disease virus (NDV) recombinants expressing infectious laryngotracheitis virus (ILTV) glycoproteins gB and gD protect chickens against ILTV and NDV challenges. J Virol 88:8397-8406. 2014.

171. Zhou, Y. C., K. Lu, S. Pfefferle, S. Bertram, I. Glowacka, C. Drosten, S. Pohlmann, and G. Simmons. A single asparagine-linked glycosylation site of the severe acute respiratory syndrome coronavirus spike glycoprotein facilitates inhibition by mannose-binding lectin through multiple mechanisms. J Virol 84:8753-8764. 2010.

172. Ziebuhr, J., E. J. Snijder, and A. E. Gorbalenya. Virus-encoded proteinases and proteolytic processing in the Nidovirales. J Gen Virol 81:853-879. 2000.

173. Ziebuhr, J., V. Thiel, and A. E. Gorbalenya. The autocatalytic release of a putative RNA virus transcription factor from its polyprotein precursor involves two paralogous papain-like proteases that cleave the same peptide bond. J Biol Chem 276:33220-33232. 2001.

174. Ziegler, A. F., B. S. Ladman, P. A. Dunn, A. Schneider, S. Davison, P. G. Miller, H. Lu, D. Weinstock, M. Salem, R. J. Eckroade, and J. Gelb. Nephropathogenic infectious bronchitis in Pennsylvania chickens 1997-2000. Avian Dis 46:847-858. 2002.