Prevention and Pathogenesis of Avian Infectious Bronchitis:
The Role of Infectious Bronchitis Virus Spike Proteins in Cell Attachment and Protective Immunity

by

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Abstract

Infectious bronchitis virus (IBV) evolves by genetic diversity and selection resulting in continuous emergence of new virus phenotypes. Spike (S) protein is responsible for virus entry. The S1 subunit of the S glycoprotein mediates viral attachment to host cells and the S2 subunit is responsible for membrane fusion. Using protein histochemistry, we investigated the tissue binding ability of the S-ectodomain of the ArkDPI-derived vaccine subpopulation previously designed C2 compared to its S1 protein alone. We demonstrated that extension of S1 with the S2 ectodomain, together comprising the S-ectodomain, greatly increases binding to chicken tissues, suggesting that vaccination with the S-ectodomain might improve protection over vaccination with S1 protein alone. Our protection study revealed that chickens immunized with recombinant S-ectodomain protein showed statistically significantly reduced viral loads 5 days post-challenge in both tears and tracheas, and exhibited improved protection of tracheal integrity compared to chickens immunized with recombinant S1 protein. These results indicate that the S2 domain has an important role in inducing protective immunity. IBV 4/91 (serotype 793/B) vaccine has been reported to protect against divergent IBV strains in a prime-boost approach with an IBV Mass vaccine. Thus, we evaluated the protective capabilities of recombinant Newcastle disease virus LaSota (rLS/IBV.S2-4/91) expressing the S2 gene of IBV 4/91, to determine whether this cross protection could be explained by cross-protective properties of 4/91 S2. We found non-significant

differences in protection 5 days post-challenge between chickens primed with Mass vaccine and boosted with rLS/IBV.S2-4/91 and chickens vaccinated with Mass only, indicating that the S2 by itself might be insufficient to induce broad cross-protective immunity. The role of S protein domains in attachment and vaccine subpopulation selection were also investigated using S proteins derived from ArkDPI-derived vaccine subpopulations with different selection pattern: C2 (strongly selected), C3 (weakly selected), and major vaccine population (negatively selected). We demonstrated that S1 proteins of vaccine virus subpopulations that are strongly positively selected in chickens bind more efficiently to chicken tissues than that of the negatively selected vaccine population. The S2 domain was required in addition to the S1 domain for binding to lung and kidney tissues. Finally, we determined the receptor binding domain (RBD) of recombinant S1 protein. The N-terminal amino acids 19-258 (NTD_{258}) of S1 is required and sufficient for binding to all chicken tissues tested except lung tissues. Only S-ectodomain bound to lung. The binding efficiency of NTD_{258} protein is better than full-length S1 protein to all tissues tested, suggesting that the remainder of the S1 protein affects the conformation of the NTD.

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List of Abbreviations

AA	Amino acid
ANOVA	Analysis of variance
Ark	Arkansas
ArkDPI	Arkansas-Delmarva Poultry Industry
C	Component
DOA	Day of age
DPC	Days post-challenge
EID_{50}	50% embryo infectious doses
ELISA	Enzyme-linked immunosorbent assay
HA	Hemagglutination
HEK293T	Human embryonic kidney 293T cells
HI	Hemagglutination inhibition
HRP	Horseradish peroxidase
IB	Infectious bronchitis
IBV	Infectious bronchitis virus
ICPI	Intracerebral pathogenicity index
MDT	Mean death time
M41	Massachusetts IBV strain 41
Mass	Massachusetts

NDV	Newcastle disease virus
PBS	Phosphate buffered saline
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
rLS	Recombinant LaSota virus
rLS/IBV.S2-4/91	rLS expressing the S2 gene of IBV 4/91 strain
S/P	Sample/positive ratio
S	Spike protein
S1	Spike S1 subunit
S2	Spike S2 subunit
Se	Spike-ectodomain
SPF	Specific-pathogen-free
$TCID_{50}$	50% tissue culture infectious dose
V	Vaccine major population

CHAPTER 1

Literature Review

1. Infectious Bronchitis (IB)

Infectious bronchitis (IB) is one of the most prevalent avian diseases that continues causing high economic impact in the world's poultry industry. In broiler chickens, IB causes reduction in weight gain and feed conversion efficiency, and increased condemnations at processing plants. In layers and breeders, it causes pronounced declines in egg production and quality [1]. Despite the use of vaccines to control IB outbreaks, the disease continues to occur in poultry farms causing production losses.

IB is a highly contagious upper respiratory tract disease [2]. Some strains affect the renal, reproductive, and digestive systems of chickens and thus, the disease pathogenesis differs according to the system involved, as well as the strain of the virus [3, 4]. Chickens of all ages and breed types are susceptible to IB, but the extent and severity of the disease is more pronounced at young ages [5-7].

IB is characterized by a high morbidity rate that can reach up to 100% while the mortality rate may range from 25 to 30% in young chicks but may increase to 80% as a result of secondary bacterial infections, mainly *Eschericia coli*, or coinfection with immunosuppressive viruses such as Marek's disease virus and infectious bursal disease virus [2]. Moreover, nephropathogenic IB strains may not produce significant

respiratory lesions or clinical signs [8] but cause high mortality, compared with strains infecting only the respiratory or reproductive systems [9]. The outcomes of infection may be also worsen due to factors that are host-associated (age, immune status), virus-associated (strain, pathogenicity, virulence, and tissue tropism), or environmental-associated (cold and heat stresses, dust, and presence of ammonia) [10]. For example, greater mortality has been observed in males, under cold stress conditions, and in certain lines [11].

IB has a short incubation period that varies with infective dose and route of infection [2]. Initial infection occurs in the host mucosal surfaces of the respiratory tract [2]. It also affects other tissues including kidney [12-14], and reproductive system (oviduct, testes) [5, 15-18]. Moreover, IB has been shown to affect the digestive system despite that it does not result in significant clinical gastrointestinal disease [4, 19].

The virus is transmitted via respiratory secretions, as well as infected poultry feces. Contaminated objects and utensils aid transmission and spread of the virus from one flock to another. The severity and clinical features of IB depend on the organ involved. Infection of the respiratory system results in clinical signs such as gasping, sneezing, tracheal rales, frothy conjunctivitis and nasal discharges [2] . Infection with nephropathogenic strains, mainly in broiler chickens, causes clinical signs including depression, wet droppings, and excessive water intake [2]. Infection of the reproductive tract is associated with lesions of the oviduct, leading to decreased egg production and quality. Eggs may appear misshapen, rough-shelled, or soft with watery egg yolk [3, 10, 20].

2. Infectious Bronchitis Coronavirus (IBV)

Infectious bronchitis virus is a member of the Coronaviridae, which includes two genera, Coronavirus and Torovirus, in the order Nidovirales [21, 22]. Coronaviruses are divided into four major groups; alphacoronaviruses (α-CoV), betacoronaviruses (β-CoV), gammacoronaviruses (γ-CoV) and deltacoronaviruses (δ-CoV) based on their phenotypic and genotypic characteristics. They are enveloped pleomorphic viruses with large club-shaped surface projections from their envelopes that give them a "corona" (crown)-like appearance. IBV is a gammacoronavirus [2, 3]. IBV was the first coronavirus described, and was discovered in the Unites States in the 1930s [23].

Coronaviruses possess a single-stranded positive-sense RNA genome that is the largest genome (26–32 kb) among RNA viruses [24, 25]. The 5′ two-thirds of the IBV genome comprises open reading frame (ORF) 1ab, which encodes 15 nonstructural replicase proteins (nsp2-16) involved in RNA replication and transcription. The 3′ one-third of the viral genome codes for the structural proteins including the spike (S), envelope, membrane, and nucleocapsid proteins [25]. The structural protein genes are interspersed by the accessory genes 3a, 3b, 4b/intergenic region, 5a, 5b [25].

The virus initially infects and replicates in the epithelium of the upper and lower respiratory tract including conjunctiva, Harderian gland [26, 27], nasal turbinates, trachea, lungs, and air sacs resulting in loss of ciliary activity, degeneration, desquamation and necrosis [2]. Then after a brief viremia, the virus may spread from the respiratory tract to the epithelial cells of the renal tubuli, causing renal failure with urate deposition and tubular necrosis [12-14], and the ciliated epithelium of the oviduct, causing necrosis and malformation [5, 15-17]. Also, IBV has been shown to replicate in

many cells of the alimentary tract (esophagus, proventriculus, duodenum, jejunum, bursa of Fabricius, cecal tonsils, rectum, and cloaca) with little pathobiological clinical effect [4, 19, 28-30]. IBV strains damage the respiratory epithelium, often predisposing young chickens to secondary bacterial infections that cause airsacculitis and systemic colibacillosis [31-33]. Laboratory studies showed that chickens inoculated with IBV and *E. coli* had more severe and persistent respiratory lesions than those inoculated with IBV alone [33-35].

The nature of the IBV persistence in tissues remains unclear. The virus has been found to commonly persist in the kidney [36], and in the alimentary tract in young chickens and layers in the absence of clinical disease [4, 19]. In addition, live vaccine strains such as the Arkansas strain have been shown to persist in the respiratory tract [26, 37, 38], especially when administered in combination with another attenuated strain (e.g., Massachusetts) [39].

3. Coronavirus Spike Protein Structure and Function

3.1. Spike Protein Structure

The spike (S) protein is the largest coronavirus structural protein (16–21 nm) and protrudes from the virion surface, giving it a corona-like appearance by electron microscopy [25, 40]. The S protein consists of four major domains: N-terminal signal sequence; the ectodomain, which is present on the outside of the virion particle; the transmembrane region responsible for anchoring the S protein into the lipid bi-layer of the virion particle; and the cytoplasmic tail (endodomain). The signal peptide directs the S protein to the endoplasmic reticulum, where it is cleaved off and the remainder of the

protein glycosylated [41]. The S protein monomer is a transmembrane glycoprotein with a molecular mass of around 128 kDa before glycosylation [25], while after glycosylation in the endoplasmatic reticulum its mass increases to about 200 kDa [42]. These protein monomers oligomerize to form trimers [40, 43]. The S ectodomain is subdivided into S1 and S2 domains, and the S1 domain further subdivided into N-terminal and C-terminal S1 domains [44].

In some coronaviruses, the S protein is cleaved by a furin-like host cell protease generating S1 and S2 subunits, while in other coronaviruses it remains in an uncleaved form [24]. The IBV S protein is cleaved into N-terminal S1 subunit and C-terminal S2 subunits of about 500 and 600 amino acids in size, respectively [2, 45-47].

3.2. Spike Protein Function

3.2.1. Attachment

The S1 subunit of S is responsible for viral attachment to cells [48-52], and the S2 domain mediates fusion of the virion envelope and cellular membranes allowing viral genomes to enter host cells [53, 54]. The receptor binding activity of the coronavirus spike protein is found to be located within the S1 domain. For a wide variety of coronaviruses, the receptor binding domain (RBD) has been identified in either the N-terminal domain (NTD) or C- terminal domain (CTD) of the S1 domain [50, 52, 55-58]. For example, murine betacoronavirus uses the NTD of S protein to bind to its receptor [59, 60], whereas the β-CoVs severe acute respiratory syndrome (SARS) and Middle East respiratory syndrome and several α-CoVs use the CTD in their S proteins to recognize their receptor proteins [61-64]. In addition, Li proposed that N-terminal RBDs

are used for mediating binding to carbohydrates whereas the C-terminal RBDs are commonly responsible for binding to protein receptors [65].

A wide range of diverse cellular receptors specifically recognized by coronavirus S1 domains have been identified. While some α- and β-CoVs require a single protein receptor for host cell attachment [56], other CoVs require co-receptors. For example, SARS CoV S protein binds to a cellular protein receptor, the angiotensin-converting enzyme (ACE-2) [66] but also uses additional host proteins for attachment including DC-SIGN, DC-SIGNR and LSECT [56, 67]. Other examples include some α-CoVs such as human coronavirus (HCoV-229E) and transmissible gastroenteritis coronavirus, which use aminopeptidase N (APN) as a receptor and also possesses the ability to bind sialic acids [56, 68, 69]. IBV uses α2,3-linked sialic acids on host cells for attachment [52, 70-72]. In addition, host calcium dependent (C-type) lectins have been recognized to play a role in infection by IBV [56]. Other host factors implicated in IBV entry include heparan sulfate, which has been shown to play a role in IBV Beaudette strain infection in vitro [73]. A protein receptor of IBV has not yet been identified [51, 70, 71].

The RBD of the IBV Massachusetts strain (M41) S protein has been mapped [50]. Protein histochemistry assays showed that the NTD of M41 S1 (AA 19–272) is required and sufficient for binding to the chicken respiratory tract in an α-2,3-sialic acid-dependent manner. In addition, comparing the binding ability between M41 and Beaudette S1 proteins identified the S1 amino acids 38, 43, 63, and 69, partly overlapping with hypervariable region 1, as critical residues for binding of M41 S1 [50].

Although S2 subunits are suggested to not contain an additional independent receptor-binding site, the interplay between S1 and S2 might synergistically determine

the avidity of virus attachment [42, 74]. For example, IBV Beaudette strain S1 was not sufficient for binding, while Beaudette S1 extended with the S2 portion of the spike ectodomain resulted in ability to bind to chicken trachea, chorioallantoic membrane (CAM) and BHK21 cells [42].

3.2.2. Host/tissue tropism and pathogenesis

S protein plays a key role in determining the species, tissue and cell tropism of coronaviruses, including IBV [52, 75-79]. For example, replacement of the spike ectodomain of Beaudette strain IBV with that of the M41 strain generates a virus with the cell tropism of M41 [80]. Recombinant avian infectious bronchitis virus expressing a heterologous spike gene demonstrates that the spike protein is a determinant of cell tropism [52, 58, 80]. For other coronaviruses such as transmissible gastroenteritis coronavirus, the enteric or respiratory tropism is determined by the spike gene, as two amino acid changes in spike protein result in the loss of enteric tropism [75]. Furthermore, spike mutations in IBV due to selective pressure in various adult chicken tissues cause a phenotypic shift [81] or reversion to virulence of vaccine strains [82, 83].

However, accumulating evidence has demonstrated that the genes outside of the S gene (nsp) may also contribute to pathogenicity of various coronaviruses [84, 85]. In the case of IBV, replacement of the spike protein of the apathogenic Beaudette strain by those of virulent M41 or 4/91 IBV strains in the viral Beaudette genome resulted in still apathogenic recombinant viruses [86, 87]. Thus, the specific role of the spike protein in determining tropism and pathogenicity remains to be elucidated since these viruses

accumulate mutations not only in the spike gene but also in other parts of the viral genome.

3.2.3. Genetic diversity

Multiple serotypes of IBV have been identified worldwide and novel serotypes/genotypes of the virus continue to arise. The most common serotype of IBV reported in the poultry industry worldwide is Massachusetts (Mass-type). In the United States, the most frequently identified serotypes are the Arkansas (Ark), Connecticut, Massachusetts, Georgia variants, and California variants [88, 89]. IBV successfully evades the extensive vaccination programs, resulting in poor control worldwide. The genetic diversity of IBV is generated by accumulated mutations made by viral RNA-dependent RNA polymerase and by recombination events followed by selection [77, 82, 90]. The RNA dependent-RNA polymerase of IBV causes point mutations in the viral genome during replication, causing high mutation rates. Recombination events may occur when co-infection with different strains of IBV occurs in the same host cell which may result in the emergence of new strains and serotypes.

Variability in the spike protein is relevant for the evolutionary success of IBV. The S1 subunit is the most variable protein and high sequence diversity of the S1 accounts for serotypical variation and IBV's immunologic escape [1, 91, 92]. Kusters et al. [92] concluded that most of the amino acid substitutions were found in the region of the first 300 N-terminal residues of S1, suggesting that serotype determinants are located in this region. Moreover, comparing the S1 sequences of six strains of related serotypes showed that most differences were found between residues 43 to 144 and residues 285 to

325 [93]. Also, Kant, et al., [94] identified mutations in neutralizing antibody escape mutants within three regions corresponding to amino acid residues 24 to 61, 132 to 149 and 291 to 398 of the S1 protein.

Additionally, the routinely used commercial attenuated live ArkDPI IBV vaccine contributes to IBV's evolutionary process. It has been shown that ArkDPI vaccine virus can persist within a flock and thus, this vaccine virus undergoes mutation and recombination with virulent field strains, resulting in emergence of novel serotypes [1, 81-83, 95]. Many field isolations of Ark-type IBV appear to be closely related genetically to ArkDPI vaccine virus [96]. In addition, distinct subpopulations in Ark DPI-derived vaccines that are rapidly positively selected in the environment of the chicken respiratory tract have been identified based on S1 gene sequences and suggested to result from mutation and selection [81-83]. The dominant IBV vaccine genotype/phenotype further changes during host invasion, likely as a result of the microenvironment of distinct tissues exerting selective pressure on the replicating viruses [81].

4. Control Strategies for Infectious Bronchitis Virus

Good biosecurity measures are important for IBV prevention. All-in/all-out operations of rearing along with cleaning and disinfection, and strict isolation are essential for IB prevention and control [2]. However, airborne transmission of the virus may complicate management control practices and render biosecurity measures ineffective. Thus, immunization remains the main strategy for control of IBV [2].

Both live attenuated and inactivated conventional vaccines are used to control IBV infection in the field. Generally, broiler type birds are vaccinated with live vaccines via drinking water or by coarse spray, while layers and breeders are primed with live vaccines, then boosted with inactivated vaccines. In the United States, broiler type birds are vaccinated with live vaccines at the hatchery, then boosted at 18-21 days of age. In layers and breeders, pullets are vaccinated with live vaccines before lay, then boosted with inactivated vaccines later. In some cases, vaccination with inactivated vaccines during lay is recommended in single age group laying flocks to reduce production losses [2].

Most of the commercially available live attenuated vaccines were derived from Massachusetts serotype strains such as M41 and the Dutch H52 and H120 strains, although some strains with regional or local impact have been used in different parts of the world [97]. Chickens that have recovered from the natural disease are resistant to challenge with the same virus (homologous protection), but the extent of protection to challenge with other IBV strains (heterologous protection) varies [2]. As discussed before, the greatest disadvantages of using live vaccines are the potential reversion of vaccine virus to virulence, and persistence of the vaccine virus in vaccinated chickens, which potentially leads to recombination events between vaccine strains and virulent field strains, leading to the emergence of new IBV serotypes [82, 83, 95, 97-99]. Moreover, vaccination with live attenuated vaccines was shown to encourage viral spread among broiler chickens [100]. Inactivated vaccines are usually used in layers and breeder chickens by injection at 13 to 18 weeks of age [101]. Since inactivated vaccines do not replicate, they cannot revert to virulence and cause pathological effects. However,

because inactivated vaccines do not replicate in the host, they induce shorter immune responses by antibody production, and no T-cell-mediated responses, compared to live attenuated vaccines [102, 103]. Therefore, inactivated vaccines in most cases require priming with live attenuated vaccines, large doses of adjuvants, and/or multiple vaccinations. Therefore, alternative vaccine strategies, such as recombinant or vector-based vaccines, are required to reduce the problems associated with vaccine reversion and thus reduce the continual introduction of new IBV serotypes.

The S1 spike subunit of IBV is the major target of virus neutralizing antibodies [94, 104] as well as an important antigen for cell-mediated immunity [102]. Most of the antigenic sites of coronaviruses that induce neutralizing antibodies are located on S1 of S [57, 59, 105, 106]. Moore et al., [107] determined that residues 304 and 386 are involved in a virus neutralizing, serotype-specific epitope on the S1 subunit of IBV using Mab-neutralization-resistant mutants. Additionally, several antigenic sites in the S1 proteins of IBV strains D207 and M41 have been identified [94, 108]. Removal of S1 but not the S2 spike protein of IBV M41 by urea resulted in failure to induce virus neutralizing antibody [45]. Thus, the S1 subunit of IBV spike protein has been suggested as a vaccine candidate.

Protection studies support the use of recombinant vaccines expressing the IBV S1 gene. The S1 sequence of IBV Vic S strain expressed from a plasmid under the control of the fowl adenovirus major late promoter induced an S1-specific antibody response and provided protection at the trachea level [109]. In addition, Toro, et al., [110] investigated the protective properties of an IBV ArkDPI S1 protein expressed from a replication-defective recombinant adenovirus vector. After virulent IBV Ark challenge,

the vaccinated chickens were protected from clinical signs, had significantly reduced challenge virus loads, and had increased IgA and IgG IBV-specific antibody-secreting lymphocytes in the spleen [110]. Similarly, a recombinant Newcastle disease virus LaSota strain expressing the S1 gene of the LX4-type IBV ck/CH/LDL/091022 induced IBV-specific IgG antibodies and cellular immunity after prime-boost vaccination [111]. Additionally, chickens vaccinated in ovo with recombinant adenovirus expressing the S1 gene of nephropathogenic IBV and boosted by an intramuscular inoculation showed a dramatic increase of both humoral and cellular immune responses [112].

In contrast, some previous studies that used recombinant S1 protein as a subunit vaccine indicated that S1 protein alone does not induce effective protection against IBV challenge [113-115]. For instance, at least four immunizations with purified S1 glycoprotein were required to induce protection against nephropathogenic IBV N1/62 strain challenge [113]. Similarly, three immunizations with KM91 S1 protein expressed by a recombinant baculovirus produced only 50% protection against virulent nephropathogenic IBV KM91 strain challenge [114]. Recombinant BacMam virus that expressed the IBV-M41 S1 glycoprotein under the control of the human cytomegalovirus immediate early enhancer/promoter provided 55% protection, and induced a significant cellular immune response in vaccinated chickens compared with inactivated vaccine [115]. However, BacMam virus displaying the S1 glycoprotein on the baculovirus envelope elicited strong humoral and cell-mediated immune responses, as well as 83% protection rate in immunized chickens following challenge with virulent IBV-M41 [116]. Furthermore, S1 protein fused to the HA2 or TM proteins of hemagglutinin of H3N2

influenza virus have been shown to provide protection rates of 87% compared with recombinant S1 protein alone (47%) [117].

IBV reverse genetics systems based mainly on cell culture-adapted Beaudette strain [118], and recently on other strains, including vaccine strains, have been used as tools for novel vaccine development. For example, recombinant IBV developed by replacing the IBV Beaudette strain S1 gene with that from the H120 strain provided 80% immune protection against challenge with virulent IBV M41 strain [119]. Similarly, Beaudette virus carrying the S1 gene of the virulent M41 IBV strain provided immune protection to chickens that were challenged with M41 [86, 87, 119, 120].

Certainly, the S1 subunit of IBV is required for attachment [3, 49-52] and the S2 subunit has traditionally been considered to play a role only in subsequent entry [53, 54]. However, a role for the S2 ectodomain in binding to cells has been demonstrated for spike proteins of Massachusetts serotype IBVs, i.e. the highly-attenuated Beaudette strain and the virulent M41 strain [42, 58]. In addition, unlike S1, the S2 portion of S is highly conserved among different IBV strains [121-123]. Monoclonal antibodies produced against the S2 protein of IBV strain M41 are able to recognize the M41, Ark-99, and Connecticut strains as well as numerous Brazilian indigenous strains [124]. Moreover, the N-terminal portion of S2 has been shown to contain immunodominant regions and neutralization epitopes [125]. Thus, S2 has been suggested to be suitable for vaccine development. Toro, et al., [122] developed recombinant Newcastle disease virus (NDV) LaSota (rLS) expressing the S2 gene of an Ark-type IBV strain. They demonstrated that chickens primed ocularly with rLS/IBV.S2 and boosted with a Massachusetts-(Mass) type attenuated vaccine were protected against challenge with

virulent IBV Ark type. This study provided evidence that overexposing the IBV S2 to the chicken immune system provides protection against challenge [122]. Based on the secondary structure S2 predictions, it has been suggested that the S2 subunit can affect binding of S1 subunit specific antibody [123] however, it has not been elucidated whether the interaction between S1 and S2 subunits could determine the shape or availability of S1 subunit specific epitopes.

Thus, we still need an understanding of the key roles of the S protein in coronavirus entry and pathogenesis that might be important for further development of control strategies. This dissertation demonstrates the role of recombinant IBV spike proteins and their domains in attachment, vaccine subpopulation selection, and generation of protective immunity.

Research Objectives:

1. Evaluate the effect of immunization with recombinant S-ectodomain protein compared to S1 protein alone on protection against IBV Ark challenge.

2. Evaluate the cross-protection conferred by a recombinant Newcastle disease virus (NDV) LaSota (rLS) expressing the S2 gene of IBV 4/91 (serotype 793/B) against Ark-type virulent challenge.

3. Characterize the binding efficiency to relevant chicken tissues of S1 and S-ectodomains of previously identified subpopulations in Arkansas (Ark) DPI-derived vaccines with positive and negative selection patterns in chickens to elucidate the role of virus attachment in vaccine subpopulation selection in chickens.

4. Identify and characterize the receptor-binding domain (RBD) of Ark-type IBV S1 and its contributions to binding efficiency to different relevant chicken tissues.

CHAPTER 2

Protection Against Infectious Bronchitis Virus by Spike Ectodomain Subunit Vaccine

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Abstract

The avian coronavirus infectious bronchitis virus (IBV) S1 subunit of the spike (S) glycoprotein mediates viral attachment to host cells and the S2 subunit is responsible for membrane fusion. Using IBV Arkansas-type (Ark) S protein histochemistry, we show that extension of S1 with the S2 ectodomain improves binding to chicken tissues. Although the S1 subunit is the major inducer of neutralizing antibodies, vaccination with S1 protein has been shown to confer inadequate protection against challenge. The demonstrated contribution of S2 ectodomain to binding to chicken tissues suggests that vaccination with the ectodomain might improve protection compared to vaccination with S1 alone. Therefore, we immunized chickens with recombinant trimeric soluble IBV Ark-type S1 or S-ectodomain protein produced from codon-optimized constructs in mammalian cells. Chickens were primed at 12 days of age with water-in-oil emulsified S1 or S-ectodomain proteins, and then boosted 21 days later. Challenge was performed with virulent Ark IBV 21 days after boost. Chickens immunized with recombinant S-ectodomain protein showed statistically significantly ($P < 0.05$) reduced viral loads 5 days post-challenge in both tears and tracheas compared to chickens immunized with recombinant S1 protein. Consistent with viral loads, significantly reduced ($P < 0.05$) tracheal mucosal thickness and tracheal lesion scores revealed that recombinant S-ectodomain protein provided improved protection of tracheal integrity compared to S1 protein. These results indicate that the S2 domain has an important role in inducing protective immunity. Thus, including the S2 domain with S1 might be promising for better viral vectored and/or subunit vaccine strategies.

1. Introduction

Infectious bronchitis virus (IBV) is a highly prevalent coronavirus of chickens that causes economic losses worldwide despite extensive vaccination. Continuous emergence of new virus serotypes results from mutation and recombination followed by selection [1]. Routinely used live-attenuated IBV vaccines, which are affected by the same evolutionary processes, not only result in vaccine-like viruses with increased virulence and persistence [1, 83], but may also contribute genetic material for recombination with other vaccine or wild virus populations. We previously identified five minor vaccine virus subpopulations selected in chickens from Arkansas-Delmarva Poultry Industry (ArkDPI)-derived IBV vaccines, designated components (C) 1-5 [81, 83]. The selection of these viral subpopulations within 3 days post-vaccination suggests they replicate better in chickens than the predominant virus population in the vaccine prior to inoculation [81, 83].

The spike (S) protein of IBV mediates viral entry into host cells [3, 56]. Its S1 subunit mediates viral attachment to host cells and induces virus-neutralizing antibodies that are important for host protective immune responses [45, 107, 126]. However, the S1 subunit shows extensive amino acid sequence variability among IBV strains, which leads to the virus's immunological escape [1, 91, 121]. The S2 subunit of S, responsible for membrane fusion, is more conserved among IBV strains [122]. The N-terminal portion of S2 contains immunodominant regions and a neutralizing epitope and therefore the S2 protein has been suggested for vaccine development [122, 125].

Previous studies indicated that the S1 protein alone does not induce effective protection against IBV challenge. For instance, at least four immunizations with purified

S1 glycoprotein were required to induce protection against nephropathogenic N1/62 strain challenge [113]. Similarly, three immunizations with KM91 S1 protein expressed by a recombinant baculovirus produced only 50% protection against virulent nephropathogenic KM91 strain challenge [114].

The S1 subunit of IBV is sufficient for attachment [3, 49-52] and the S2 portion of coronavirus spike proteins has traditionally been considered to play a role only in subsequent entry [53, 54]. However, a role for the S2 ectodomain in binding to cells has been demonstrated for spike proteins of Massachusetts serotype IBVs, i.e. the highly-attenuated Beaudette strain and the virulent M41 strain [42, 58]. In the current study, we evaluated binding of trimeric Ark S-ectodomain compared to trimeric S1 subunit alone to multiple relevant chicken tissues. After confirming improved binding of Ark S-ectodomain, which might be explained by the presence of the S2 ectodomain altering the conformation of S1 and thus increasing its affinity for receptors, or by S2 directly contributing to interaction with receptors or co-receptors, we tested the hypothesis that immunization with recombinant soluble trimeric S-ectodomain provides more effective protection than immunization with trimeric S1 subunit alone.

2. Materials and methods

2.1. Genes and expression vectors

The amino acid sequence of S proteins representing an IBV ArkDPI vaccine subpopulation previously designated C2 (GenBank accession ABY66333) was chosen to produce recombinant proteins. C2 was strongly selected in chickens after vaccination with an ArkDPI-derived attenuated vaccine [81, 83]. Its S1 is almost identical to that of

the unattenuated parent ArkDPI isolate [127] and represents the consensus sequence of vaccine subpopulations rapidly positively selected in chickens after vaccination with ArkDPI-derived attenuated vaccines [1, 81-83, 128]. To generate recombinant S1 protein, a human codon-optimized sequence encoding C2 S1 [amino acids (AA) 19-538] was synthesized (GeneArt, Regensburg, Germany) and cloned into the pCD5 vector. To generate recombinant S-ectodomain, a human-codon optimized sequence encoding the C2 S2 ectodomain (S AA 544-1097) was cloned into the pCD5 vector already containing the S1 domain as described [42]. At the S1/S2 border, the furin cleavage site sequence RRSRR was replaced by GGGVP to avoid cleavage of the full length S-ectodomain [42]. These S1 and S-ectodomain-coding sequences were flanked by sequences encoding an N-terminal CD5 signal sequence and sequences encoding C-terminal artificial GCN4 trimerization motif and Strep-tag II for purification and detection of proteins, as described [52] .

2.2. Recombinant S protein production and purification

Soluble trimeric recombinant S1 and S-ectodomain proteins were produced in human embryonic kidney (HEK) 293T cells as described [42, 52, 129]. In brief, the expression vectors encoding S1 or S-ectodomain were transfected into HEK293T cells and recombinant proteins purified from tissue culture supernatants 6 days post-transfection using Strep-Tactin® Sepharose columns according to the manufacturer's instructions (IBA GmbH, Göttingen, Germany). The concentration of purified proteins was determined by Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA). The purified

proteins were confirmed and concentrations normalized by electrophoresis in Mini-PROTEAN®TGX Stain-Free™ Precast Gels (Bio-Rad, Hercules, CA).

2.3. Binding to tissues by protein histochemistry

The binding efficiency of S1 and S-ectodomain proteins to tissue sections prepared from healthy specific pathogen free (SPF) 40-day old white leghorn chickens was assessed by protein histochemistry as described [42, 129] with minor modifications: antigen retrieval was conducted at 80° C for 30 min, Tris buffers were substituted for phosphate buffers, slides were blocked with universal negative serum (Biocare, Pacheco, CA) instead of 10% goat serum, and the addition of most reagents and washing steps were performed by an intelliPATH FLX automated slide stainer (Biocare, Pacheco, CA). S proteins and 3-amino-9-ethyl-carbazole (AEC+; Dako, Carpinteria, CA) were added manually. Briefly, S proteins (100 µg/ml for S1 and 50 µg/ml for S-ectodomain) pre-complexed with Strep-Tactin-HRP (IBA GmbH, Göttingen, Germany) were incubated with deparaffinized and rehydrated tissue sections overnight at 4 °C. Bound S protein was visualized with AEC+ chromogenic substrate. The tissues were counterstained with hematoxylin and mounted with Lerner AquaMount (Covance, Princeton, NJ). Images were captured from an Olympus BX41 microscope with an Olympus DP71 12 mp camera.

2.4. Protection trial

2.4.1. Chickens

White leghorn chickens hatched from SPF eggs (Charles River, North Franklin, CT) were 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 guidelines. Auburn University College of Veterinary Medicine is an Association for Assessment and Accreditation of Laboratory Animal Care-accredited institution.

2.4.2. Experimental design

Four groups of chickens (each *n*=16-17) were used. Chickens were primed at 12 days of age (DOA) by subcutaneous injection in the neck region of 0.2 ml containing 10 µg of S1 (group A) or 20 µg of S-ectodomain protein (group B) emulsified in Montanide™ ISA 71 VG adjuvant (Seppic, Paris, France). Twice the amount of S-ectodomain protein was used because recombinant S-ectodomain is 1.96-times the molecular weight of recombinant S1. Thus, approximately equimolar amounts of protein were administered. Chickens in groups A and B were subsequently boosted with the same adjuvanted protein 21 days later. Control group C (non-vaccinated) was primed and boosted with PBS and the adjuvant, and group D was the unvaccinated/unchallenged control group. Chickens in groups A, B and C were challenged 21 days after boost by ocular and nasal instillation of 10^5 50% embryo infective doses (EID_{50}) of a virulent IBV Ark-type strain (GenBank accession JN861120) previously characterized [18]. Protection was evaluated 5 days post-challenge (DPC) by viral load in tears and tracheas, tracheal

histomorphometry, and tracheal histopathology lesion scoring. In addition, antibodies in sera specific for IBV or S protein were determined by ELISA before prime (11 DOA), three weeks after prime (32 DOA), two weeks after boost (45 DOA) and 5 days post-challenge.

2.4.3. Viral load by qRT-PCR

Relative IBV RNA levels in tears and tracheas were determined by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Viral RNA was extracted from individual tear samples using the QIAmp viral RNA mini kit (Qiagen, Valencia, CA), and from homogenized tracheas with TriReagent® RNA/DNA/protein isolation reagent (Molecular Research Center, Cincinnati, OH) following the manufacturers' protocols. Relative viral RNA concentrations in tear and tracheal samples were determined by TaqMan® qRT-PCR as described [130]. Data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple comparisons post-test.

2.4.4. Tracheal histomorphometry and histopathology

Histomorphometry of the tracheal mucosa was evaluated blindly as described [89]. Briefly, formalin-fixed sections of trachea collected from challenged and control birds at 5 days post-challenge 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. The severity of lesions (tracheal deciliation and epithelial

necrosis) was scored (1=normal, 2=mild, 3=moderate, 4=marked, 5=severe) and the average of the two scores determined as a lesion score for each chicken. Histomorphometric data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons post-test. Lesion scores were analyzed by Kruskal-Wallis test followed by Dunn's multiple comparisons post-test.

2.4.5. Antibodies measured by ELISA

2.4.5.1. IBV-specific ELISA.

IBV-specific ELISA was performed as previously described [131]. Briefly, ELISA plates (Nunc MaxiSorp Immuno Plates; Thermo Scientific) were coated with heat-inactivated IBV (ArkDPI vaccine strain; S AA sequence GenBank #ABY66334) purified as described [131]. Individual chicken sera diluted 1:100 were loaded and plates incubated at 4 °C overnight. IBV-specific IgG was detected using biotinylated monoclonal mouse–anti chicken IgG [(clone G-1) Southern Biotechnology Associates, Inc., Birmingham, AL], streptavidin-conjugated HRP (Southern Biotechnology Associates, Inc.) and tetramethylbenzidine (TMB; Invitrogen Corp., Frederick, MD) HRP substrate. Absorbance at 450 nm was measured with a Powerwave XS (BioTek Instruments, Inc., Winooski, VT).

2.4.5.2. S1 and S-ectodomain protein-specific ELISA.

ELISA plates (Nunc MaxiSorp Immuno Plates; Thermo Scientific) were coated with 100 µl of 0.25 µg/ml of either recombinant S1 protein or S-ectodomain protein at 4 °C overnight. Plates were drained and blocked with 200 µl of 1% bovine serum albumin

and 0.05% Tween 20 in PBS for 1 h at room temperature. Plates were drained and individual chicken sera (diluted 1:100) were loaded and incubated 30 min at room temperature. Plates were washed and antibodies detected using reagents in a commercial IBV ELISA kit (Idexx Laboratories, Inc., Westbrook, ME) following instructions in the kit. Absorbance at 650 nm was measured with a Powerwave XS. Statistical analyses were performed using one-way ANOVA followed by Tukey's multiple comparisons test.

3. Results

3.1. S binding to tissues

The binding affinity of recombinant S-ectodomain to relevant chicken tissues was compared to that of recombinant S1 protein using protein histochemistry. As seen in Fig. 2.1, the S1 protein bound weakly to the epithelium of trachea, nasal mucosa, choana (not shown), cecal tonsils, and cloaca, and to secretory cells of trachea, nasal mucosa, and choana, while binding was not detected in the lung and kidney. Extension of S1 with S2 subunit ectodomain (S-ectodomain) increased binding affinity to trachea, choana, nasal mucosa, cloaca, and cecal tonsils and enabled binding to lung and kidney. It should be noted that the molar concentration of S-ectodomain used for spike histochemistry was approximately one-fourth that of S1, indicating that the binding affinity of S-ectodomain is much greater than that of S1.

3.2. Viral load

Chickens immunized with recombinant S-ectodomain protein showed statistically significant ($P < 0.05$) reductions of viral RNA both in tears and tracheas 5 days post-

challenge compared to chickens immunized with recombinant S1 protein or adjuvant alone (Fig. 2. 2). A significant ($P < 0.05$) reduction of the viral RNA in the S1-immunized group compared to mock-vaccinated chickens was detected only in tears. S1-protein immunization did not significantly reduce viral RNA levels in trachea.

3.3. Tracheal histomorphometry and histopathology

Consistent with the viral load results, the S-ectodomain-immunized chickens showed a significant reduction ($P < 0.05$) of tracheal mucosal thickness, lymphocyte infiltration, and lesion severity (tracheal deciliation and epithelial necrosis) 5 days post-challenge compared to recombinant S1 protein alone-immunized and adjuvant-only chickens (Fig. 2. 3). In contrast, no significant differences ($P < 0.05$) were detected between recombinant S1 protein-immunized and adjuvant-only groups. Remarkably, no significant differences in any of the tested tracheal histopathology parameters were detected between chickens immunized with S-ectodomain protein and unvaccinated/unchallenged controls, indicating that immunization with recombinant S-ectodomain protein provided complete protection of tracheal integrity.

3.4. Antibodies

Chickens immunized with S-ectodomain protein showed significant ($P < 0.05$) increases in IBV-specific antibodies in sera compared to those immunized with S1 protein alone and the non-vaccinated controls before challenge at 32 and 45 DOA, as well as 5 DPC (Fig. 2. 4A). However, no significant differences were detected between S1 protein-immunized chickens and non-vaccinated controls. Consistent with IBV-specific

antibodies, S-ectodomain protein-specific ELISA also revealed significant differences between the S-ectodomain protein-immunized group and the S1 protein-immunized group at all times post-immunization (Fig. 2. 4B). S1 protein-specific ELISA did not indicate any significant differences between the chickens immunized with S-ectodomain protein compared to chickens immunized with S1 protein alone (not shown). Collectively, these results indicate the presence of antibodies directed against S2 and/or S-ectodomain-specific conformational epitope(s) in chickens immunized with S-ectodomain protein.

4. Discussion

The evolutionary success of IBV and the problems associated with use of live-attenuated vaccines indicate an urgent need to develop novel vaccines. Alternative approaches such as subunit vaccines or viral-vectored vaccines expressing specific proteins would eliminate emergence of vaccine subpopulations and facilitate the rapid development of effective vaccines against new serotypes. We have demonstrated that trimeric S-ectodomain provides more effective protection than trimeric S1protein.

Comparing the binding of recombinant S1 and S-ectodomain proteins of IBV Ark-type strain revealed that S-ectodomain shows increased binding affinity to chicken tissues including trachea, choana, nasal mucosa, cecal tonsils and cloaca. Interestingly, S1 protein was unable to bind to lung and kidney tissues, which are also target organs for IBV, and required the S2 ectodomain to bind. These results are consistent with reports by others, showing that while the S1 subunit of the embryo- and cell-culture-adapted Beaudette strain is unable to bind to chorioallantoic membrane, the Beaudette S-ectodomain binds efficiently [42]. Furthermore, the extension of the M41 S1 with the

M41 S2 ectodomain domain increased binding to chicken trachea [58]. The M41 S1 shows only 77% amino acid sequence identity with the ArkDPI S1 used herein. Thus, the current results confirm these findings for another IBV serotype and additional tissues. Using chimeric S-ectodomain proteins, Promunktod et al. concluded that S2 does not contain an additional independent receptor binding site that would explain its contribution to the affinity of S for receptors [42]. Another possible explanation for improved tissue binding of S-ectodomain is that the S2 subunit is necessary for the S1 protein to adopt a conformation optimal for binding. Structures of trimeric S-ectodomains of other coronaviruses determined by cryo-electron microscopy, e.g. [44, 132], suggest that the trimeric structure is important for the conformation of S1, because the S1 domains of the monomers are interwoven in the trimer. In the recombinant S1 protein used in this study, the artificial trimerization domain immediately follows the S1 domain and could thus artificially constrain the trimeric S1 in a suboptimal conformation. When the S2 ectodomain is included between S1 and the trimerization domain, the trimers might be closer to their normal conformation. However, our unpublished results indicate that a single amino acid change in the S2 domain can reduce the binding of the S-ectodomain (S. Farjana et al., unpublished results). Thus, S2 may influence the conformation of S1 in a more specific way.

Most IBV neutralizing antibodies recognize conformational epitopes in S1 [94, 121, 126, 133]. Thus, if the S2 ectodomain allows S1 to adopt a conformation optimal for attachment, antibodies generated against this conformation might more effectively neutralize virus than antibodies generated against the suboptimal conformation of S1 adopted in the absence of S2. Therefore, we considered the possibility that extension of

recombinant S1 protein with the S2 ectodomain would improve the protection afforded by a subunit vaccine. Indeed, our protection trial results indicated that immunization with trimeric S-ectodomain protein significantly reduces viral loads in tears and trachea, as well as tracheal damage, compared to immunization with trimeric S1 protein. Moreover, there were no significant differences in tracheal damage between chickens immunized with S-ectodomain protein and unvaccinated/unchallenged control chickens, indicating complete protection. Conversely, no significant differences were observed between chickens immunized with S1 protein and the mock-immunized group except for the viral load in tears. This limited protection conferred by S1 protein is in agreement with results of others [113] who found that at least four immunizations with the purified S1 glycoprotein of nephropathogenic N1/62 strain of IBV were necessary to induce protection, even though they used a considerably larger amount of purified S1 antigen (50 µg) for immunization.

One possible explanation for improved protection following immunization with S-ectodomain, as already mentioned, is that antibodies produced to S1 in the ectodomain conformation neutralize the challenge virus more effectively than antibodies produced to S1 protein alone. Alternatively, the conserved immunodominant linear neutralizing epitope within S2 [125] might also contribute to improved protection. Although we did not attempt to demonstrate neutralizing antibodies, our ELISA results using both purified IBV and S-ectodomain protein showed a significant increase of antibody level in chickens immunized with S-ectodomain protein compared to those immunized with S1 protein alone, indicating that antibodies to S2 epitopes were generated. Furthermore, a peptide near the amino terminal end of S2 has been shown to induce a protective cell-

mediated response [134]. The adjuvant used has been reported to stimulate both antibody and cell-mediated immune responses [135-137]. The addition of the HA2 domain of the influenza hemagglutinin has also been demonstrated to increase the immunogenicity and protective capacity of IBV S1, possibly by increasing thermostability [117].

The findings that recombinant S-ectodomain protein shows improved binding to cell receptors and elicits improved protection against challenge suggests that the S2 domain has an important role in inducing protective immunity. Thus, including the S2 ectodomain with S1 provides a promising option for a subunit vaccine and expands options for better viral vectored vaccines.

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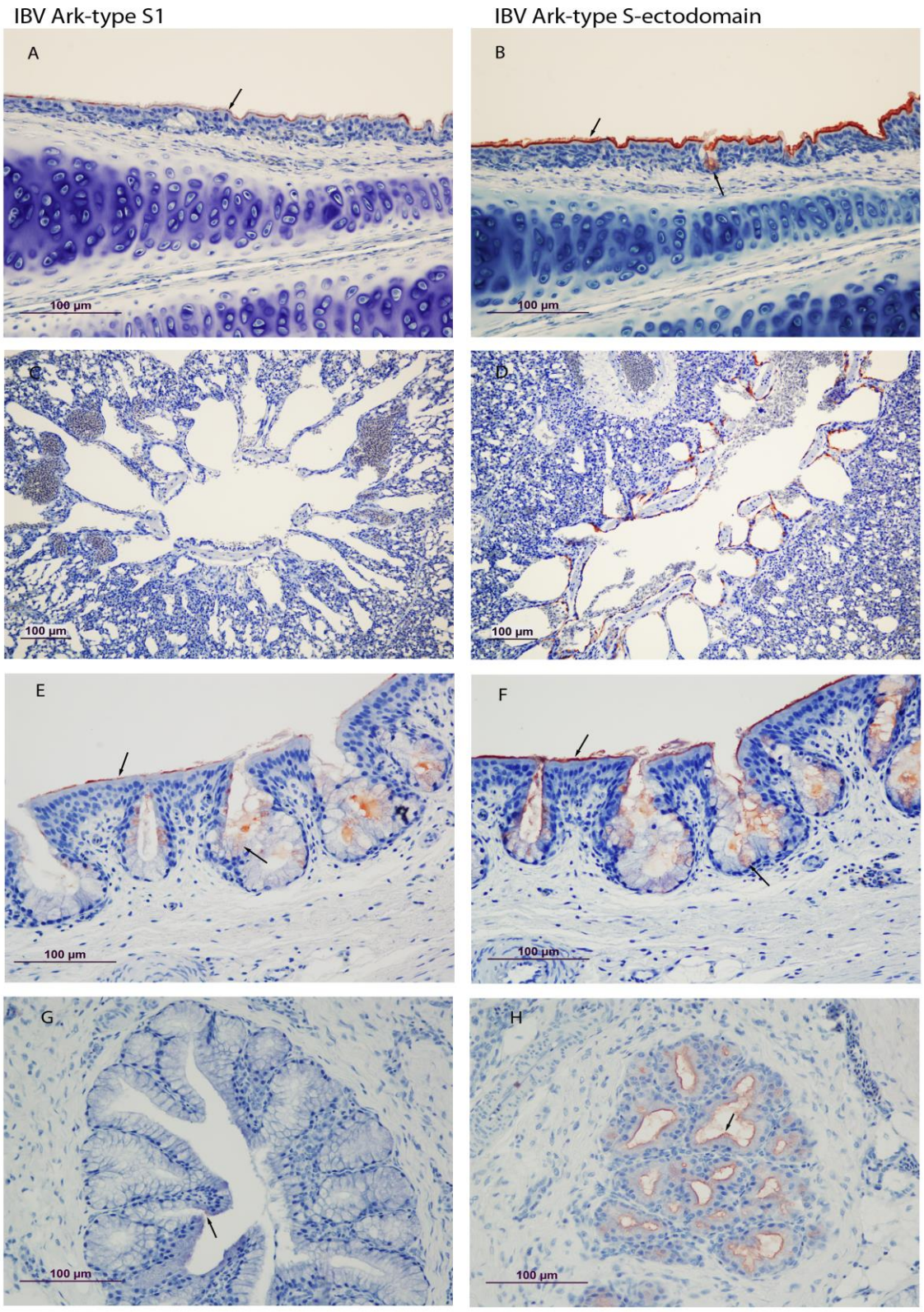
Conflict of interest statement

The authors have no conflict of interest to declare.


IBV Ark-type S1
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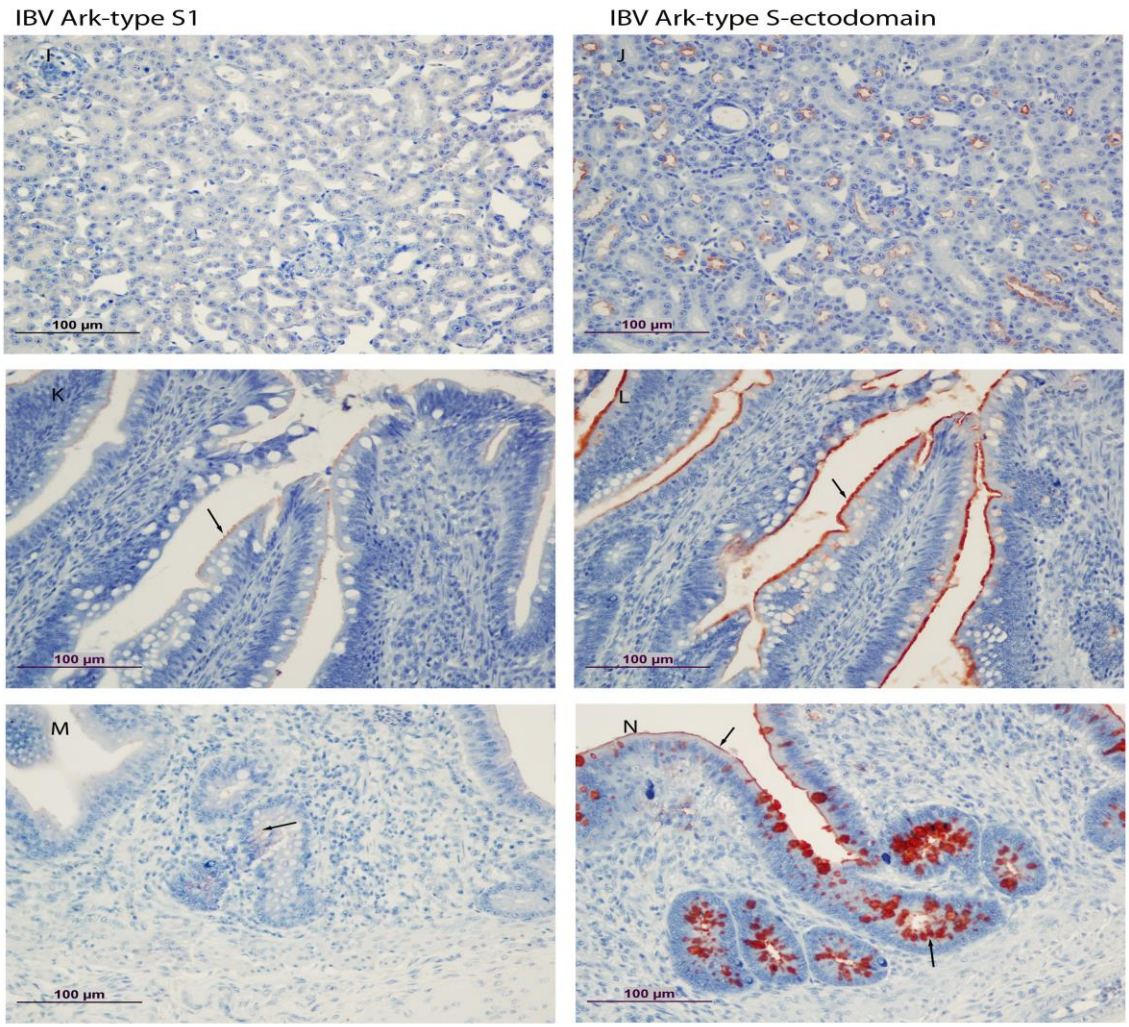


Fig. 2. 1. Protein histochemistry demonstrating recombinant IBV Ark-type S1 and S-ectodomain binding in various chicken tissues. AEC+ chromogenic substrate was used to identify bound spike protein as indicated by red staining (arrows). (A) S1 ectodomain binds to the apical surface of the tracheal epithelium, while S-ectodomain (B) binds to the

cilia, tracheal epithelium, and the mucin-containing goblet cell secretory vesicles. (C) S1 protein binding was not identified in the lung. (D) S-ectodomain binding was recognized in the epithelium lining the pulmonary parabronchi and atria. (E) Minimal staining at the apical surface of the nasal mucosal epithelium and mucus glands with S1 protein was observed, whereas S-ectodomain (F) exhibited enhanced staining of the nasal mucosal epithelium and mucus glands. (G) Sparse punctate S1 binding occurred along the apical surface of the choanal submucosal glandular epithelium. (H) Intense S-ectodomain binding of the epithelial apical surface and secretory product in the choanal glands was recognized. (I and J) S-ectodomain binding was detected on the epithelial apex of scattered renal tubules; however, S1 binding was not observed. (K) Multifocal, weak staining at the apical surface of the cecal tonsil intestinal epithelium with S1 protein was observed, whereas S-ectodomain (L) exhibited diffuse enhanced staining of the epithelium. (M and N) There was diffuse, strong staining of the cloacal glands with S-ectodomain, and only scattered, weak staining with the S1 protein. Although recombinant S-ectodomain is twice the molecular weight of recombinant S1 protein, it was used at half the μg/ml concentration. Thus the molar concentration of S-ectodomain was one-fourth that of S1. Thus, the increased binding affinity of S-ectodomain compared to S1 is even greater than it appears.

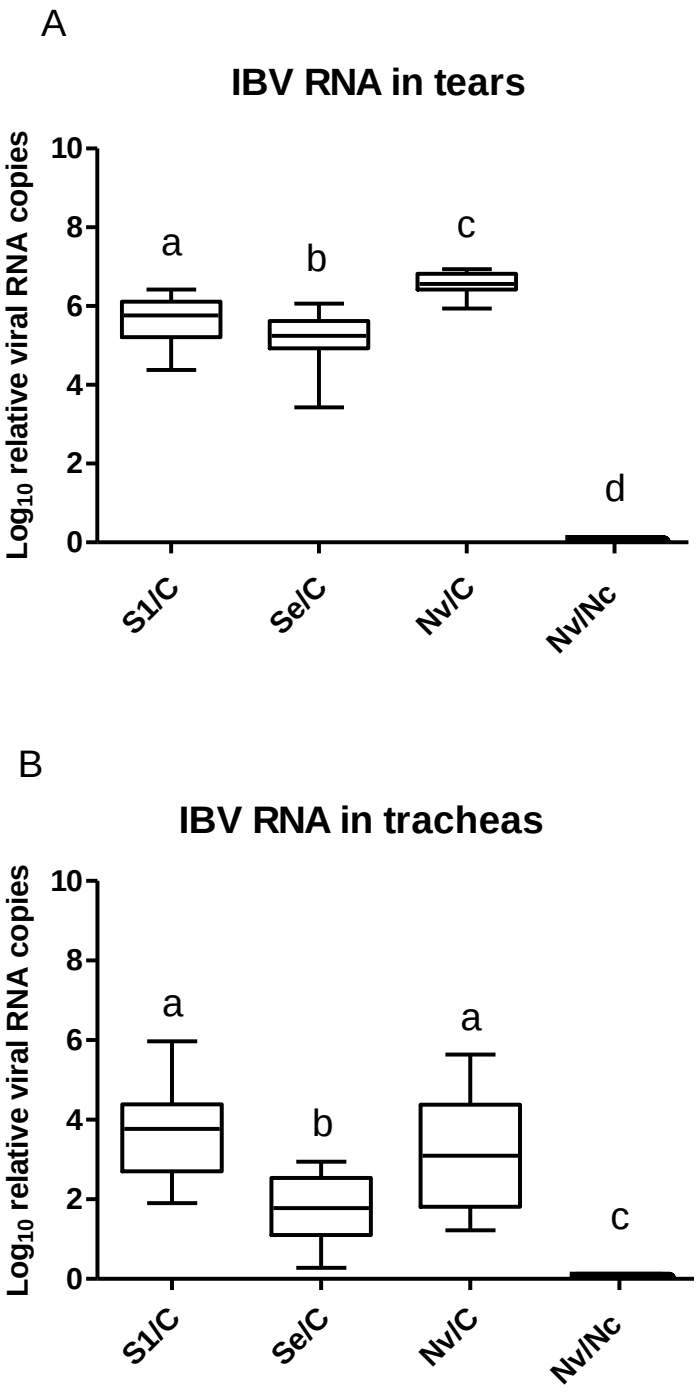
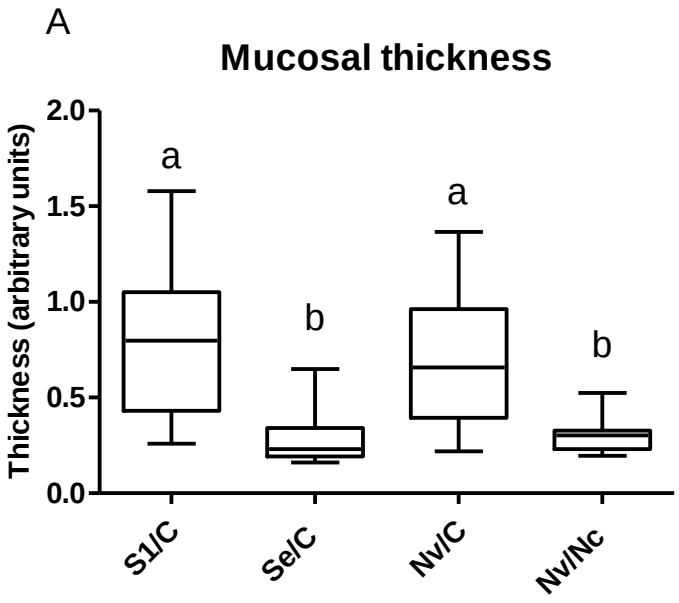


Fig. 2. 2. Relative IBV RNA in (A) tears and (B) trachea of chickens primed at day 12 of age with adjuvanted trimeric recombinant S1, or S-ectodomain (Se), boosted 21 days later, and challenged with virulent Ark-type IBV 21 days post-boost. Nv/C =non-

vaccinated (chickens primed and boosted with the adjuvant with PBS)/challenged. Nv/Nc =non-vaccinated/non-challenged. Relative IBV RNA levels determined 5 days post-challenge by qRT-PCR. Lines indicate median $\log_{10}$ relative RNA copy numbers, boxes indicate 25th to 75th percentile, and whiskers indicate minimum and maximum values. Different letters indicate significant differences ($P < 0.05$). Nv/Nc were assigned $\log_{10}$ values of 0 to be included in the graphs with log scale Y axes.



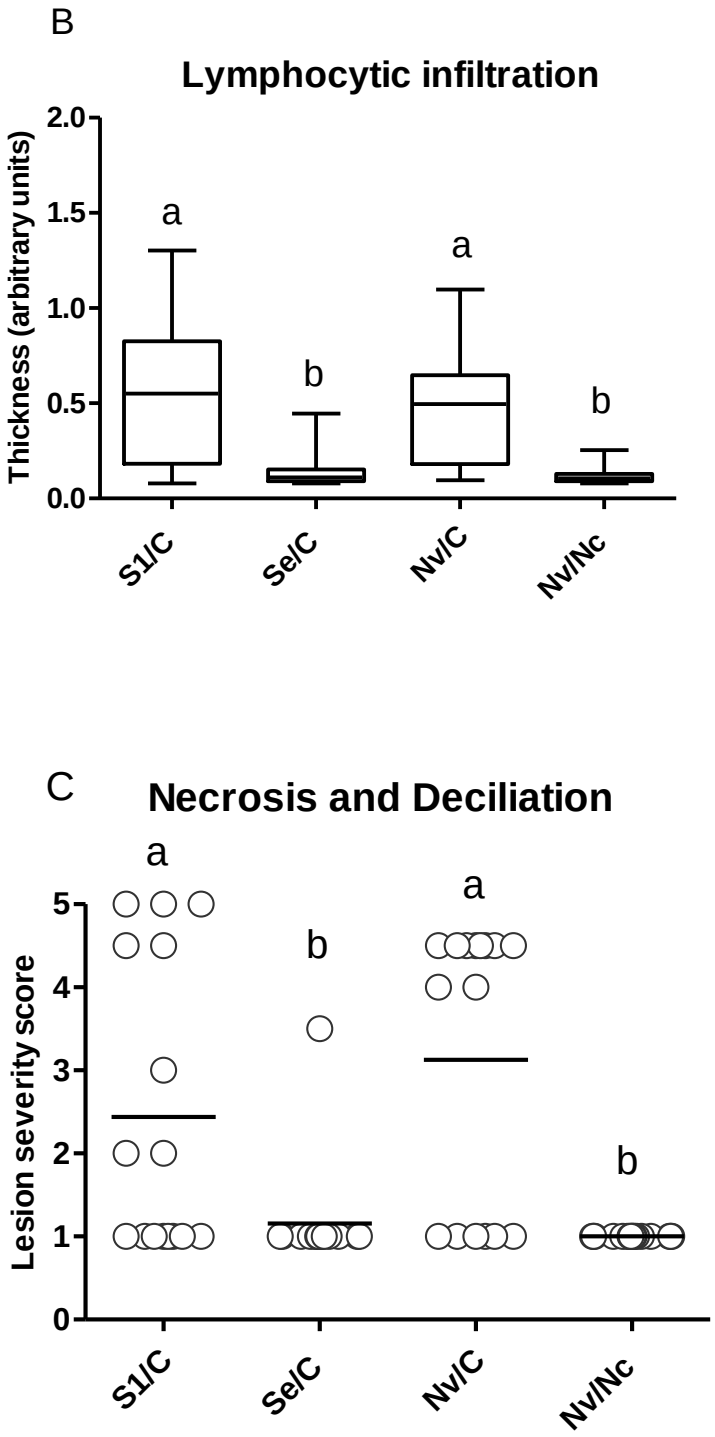
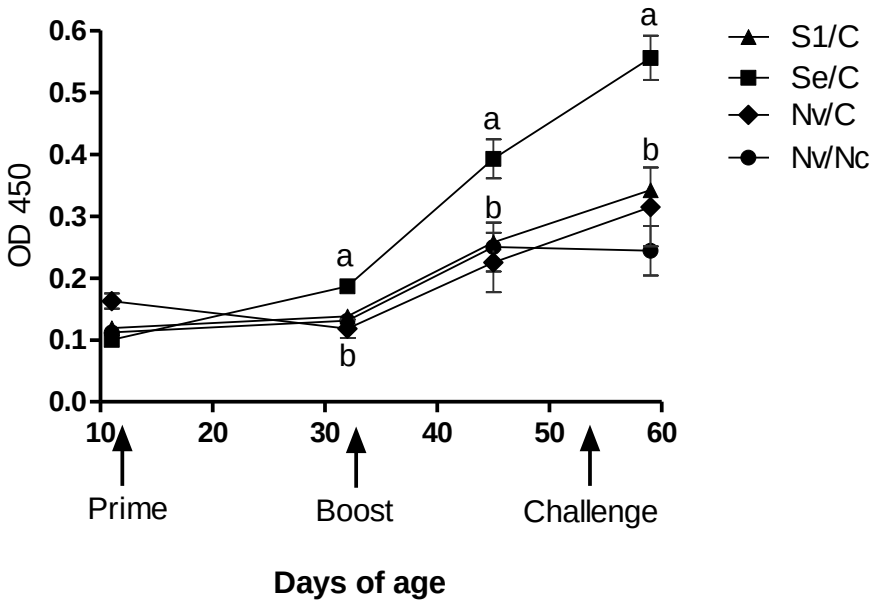


Fig. 2. 3. Tracheal histomorphometry and histopathology 5 days after virulent IBV Ark challenge in chickens primed with adjuvanted trimeric recombinant S1, or S-ectodomain

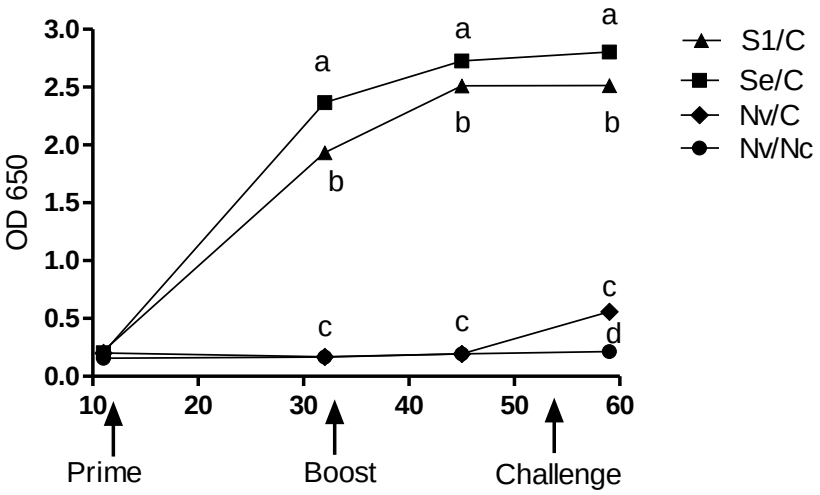
(Se), boosted 21 days later, and challenged with virulent Ark-type IBV 21 days post-boost. (A) Mucosal thickness and (B) thickness of lymphocytic infiltration by tracheal histomorphometry. (C) Severity of tracheal mucosal necrosis and deciliation scored blindly (1=normal, 2=mild, 3=moderate, 4=marked, 5=severe) for each chicken. In box and whisker plots (A and B), lines indicate the median thickness, the boxes indicate the 25^{th} and 75^{th} percentiles, and the whiskers indicate minimum and maximum values. In the scatter plot (C), each point indicates the lesion score for an individual chicken and the lines indicate mean scores for each group. Nv/C =non-vaccinated (chickens primed and boosted with the adjuvant with PBS)/challenged. Nv/Nc =non-vaccinated/non-challenged. Different letters indicate significant differences ($P < 0.05$).

A



Days of age

B



Days of age

Fig. 2. 4. IBV antibodies in chicken sera before prime (11 DOA), 3 weeks after prime (32 DOA), 2 weeks after boost (45 DOA) and 5 days post-challenge (59 DOA) determined by ELISA. IBV-specific ELISA (A), and S-ectodomain protein-specific ELISA (B) of chickens primed with recombinant S1, S-ectodomain (Se), boosted 21 days later, and challenged 21 days post-boost. Nv/C =non-vaccinated (chickens primed and boosted with the adjuvant with PBS)/challenged. Nv/Nc =non-vaccinated/non-challenged. Mean absorbance values and SEM are shown. In (B) the error bars are so small that they are obscured by the symbols. The S-ectodomain- immunized group showed significantly higher antibody levels (as measured by optical density) than the S1 protein-immunized, adjuvant only, and non-vaccinated non-challenged groups ($P < 0.05$). Statistically significant differences for each time-point post-vaccination indicated by letters.

CHAPTER 3

Infectious Bronchitis Virus S2 of 4/91 Expressed from Recombinant Virus Does Not Protect Against Ark-type Challenge

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Abstract

We previously demonstrated that chickens primed with a recombinant Newcastle disease virus (NDV) LaSota (rLS) expressing the S2 gene of IBV and boosted with an attenuated IBV Massachusetts (Mass)-type vaccine were protected against IBV Arkansas (Ark)-type virulent challenge. A possible basis for the reported ability of IBV 4/91 (serotype 793/B) vaccine to protect against divergent IBV strains (e.g. QX, Q1, D1466) in a prime-boost approach with an IBV Mass vaccine is that an immune response against the S2 protein of IBV 4/91 is cross-protective. Therefore, we evaluated the protective capabilities of the S2 protein of IBV 4/91 expressed from rLS. The level of S2 amino acid sequence identity between 4/91 and the Ark challenge strain used in this study (90.7%) is within the range of S2 amino acid sequence identities between 4/91 and Q1 (91-94%) and QX strains (89-94%). Chickens primed with attenuated Mass IBV at 1 day of age and boosted with rLS/IBV.S2-4/91 at 14 days of age were challenged with a virulent Ark IBV strain at 28 days of age. Protection (reduction of clinical signs and viral loads) assessed 5 days post-challenge showed non-significant differences between chickens primed with Mass vaccine and boosted with rLS/IBV.S2-4/91 and chickens vaccinated with Mass only. Thus, the observed level of protection is attributable only to the effect of the Mass vaccine indicating that the S2 of IBV 4/91 does not induce broad cross-protective immunity.

1. Introduction

Infectious bronchitis virus (IBV) evolves by generation of genetic diversity (mutations and recombination) followed by natural selection resulting in continuous emergence of new virus phenotypes [1]. Routinely used live attenuated vaccines are subject to the same evolutionary mechanisms and thus also contribute to the emergence of novel IBV variants [83]. The spike (S) protein of IBV mediates viral attachment and entry to host cells [40, 138-140]. Successful immunological escape is mainly due to extensive amino acid sequence variability of the S1 subunit among IBV strains [1, 92, 121]. The S2 subunit of S, responsible for membrane fusion, is more conserved among IBV strains [122]. The N terminal portion of S2 has been shown to contain immunodominant regions and neutralizing epitopes [92, 104, 125], suggesting the suitability of S2 for vaccine development. We previously developed recombinant Newcastle disease virus (NDV) LaSota (rLS) expressing the S2 gene of an Ark-type strain. Chickens primed ocularly with rLS/IBV.S2 and boosted with a Massachusetts-(Mass) type attenuated vaccine were protected against challenge with virulent IBV Arkansas (Ark)-type. Chickens vaccinated only with the recombinant virus were not protected. These results provided evidence that overexposing the IBV S2 to the chicken immune system provides protection against challenge [122].

Cook et al., [141] first showed that the attenuated IBV 4/91 vaccine strain (belonging to serotype 793B) when used in combination with an attenuated Mass- type vaccine in a prime-boost regime (Mass followed by 4/91) confers protection to heterologous serotype IBV challenge. Reports by others followed showing that this protocol effectively protected against the Q1 variant circulating in South America [142, 143], and QX-like

variants circulating in China and European countries [144]. Subsequently, the IBV 4/91 vaccine was introduced in South American countries as well as countries in the Middle East. Interestingly, the S1 amino acid sequence of IBV 4/91 differs significantly from the S1 of all strains against which reports indicate it protects. There are several possible explanations for this phenomenon. One of them is that the conserved S2 protein might be playing a role in inducing cross- protection.

In the current study, we developed rLS expressing the S2 gene of IBV 4/91 (serotype 793/B) and evaluated possible protection against Ark-type virulent challenge. The level of S2 amino acid sequence identity between 4/91 and the Ark challenge strain used in this study (90.7%) is within the range of S2 amino acid sequence identities between 4/91 and Q1 (91-94%) and QX strains (89-94%).

2. Materials and methods

2.1. Generation of rLS expressing the S2 gene of IBV 4/91 (rLS/IBV.S2-4/91)

The complete S2 gene sequence of the IBV UK4/91 (GenBank accession AEL97578.1) was optimized to chicken codons and synthesized (GenScript, Piscataway, NJ). The codon-optimized IBV S2 gene flanked by NDV gene start and gene end sequences was inserted into the rLS vector between the phosphoprotein and matrix genes as an additional transcription unit using the In-Fusion® PCR cloning kit (Clontech, Mountain View, CA) and recombinant virus generated as previously described [122, 145, 146].

2.2. Biological assessment of rLS/IBV.S2-4/91 virus

rLS/IBV.S2-4/91 was titrated in embryonated eggs as accepted [147]. The pathogenicity of the recombinant virus construct was assessed by standard mean death time (MDT) in embryos and intracerebral pathogenicity index (ICPI) in day-old chickens [122, 146].

2.3. Chickens

White leghorn chickens hatched from specific pathogen free (SPF) eggs (Charles River, North Franklin, CT) were 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 accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

2.4. Protection trial

Four groups (each n=23-25) of chickens were used. Chickens in groups 1 and 2 were ocularly vaccinated with $10^{5.0}$ 50% embryo infectious dose (EID_{50}) per bird of a commercial attenuated IBV Mass vaccine on day 1 of age. Chickens of group 1 were ocularly boosted at 14 days of age with $10^{6.0}$ EID_{50} of rLS/IBV.S2-4/91. Additional groups (3 and 4) constituted the unvaccinated/challenged and unvaccinated/non-challenged controls, respectively. Challenge was performed with $10^{5.0}$ EID_{50} of a virulent IBV Ark-type strain (GenBank accession JN861120) previously characterized [18] by ocular and nasal instillation at 28 days of age. Protection was evaluated by respiratory

signs and viral load in both tears (from all birds) and tracheas (from 15 birds per group) 5 days after challenge. In addition, serum antibodies were determined by ELISA in all birds before challenge and from the remaining chickens (n=8-10) of each group at 16 and 26 days post-challenge (DPC).

2.4.1. Clinical signs.

Respiratory signs (tracheal and nasal rales) were assessed blindly and individually, and severity scored from 0 to 3 (0=absent, 1= mild, 2=moderate, 3=severe) as previously described [122].

2.4.2. Viral load

Relative viral loads were determined by quantitation of viral RNA by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Viral RNA was extracted from individual tear samples using the Qiagen QIAmp viral RNA mini kit (Qiagen, Valencia, CA), and from the tracheas with TriReagent® RNA/DNA/protein isolation reagent (Molecular Research Center, Cincinnati, OH) following the manufacturer's protocols. The relative viral RNA concentrations in tear and tracheal samples were determined by TaqMan® qRT-PCR as described [148]. Data were analyzed by one-way analysis of variance followed by Tukey multiple comparisons post-test.

2.4.3. Antibodies

Sera collected from all chickens in each group were tested for NDV-specific antibodies by hemagglutination inhibition (HI) test as described [149], and for IBV

antibodies using a commercial IBV ELISA kit (Idexx Laboratories, Inc., Westbrook, ME).

3. Results

3.1. Biological properties of rLS/IBV.S2-4/91

The recombinant rLS/IBV.S2-4/91 showed MDT and ICPI values similar to the parental LaSota strain. MDT increased from 110 for LaSota to 144 hours for rLS/IBV.S2-4/91 and ICPI increased slightly from 0.15 (LaSota) to 0.19 (rLS/IBV.S2-4/91). The titers achieved by rLS/IBV.S2-4/91 in allantoic fluid of embryonated eggs determined by hemagglutinating activity, EID_{50}, and $TCID_{50}$ are shown in Table 3.1. Chickens boosted with rLS/IBV.S2-4/91 showed mean HI antibody titers of 2.66 ± 0.38 before IBV challenge. HI titers detected on days 16 and 26 after challenge were 2.33 ± 0.52, and 2.37 ± 0.67, respectively. Chickens of groups not exposed to rLS maintained a negative NDV antibody status.

3.2. Protection trial

As seen in Fig. 3.1, both vaccinated groups (1 and 2) showed significantly less severe respiratory signs than unvaccinated/challenged controls (group 3). No significant differences ($P < 0.05$) were detected between chickens primed with Mass and boosted with rLS/IBV.S2-4/91 (group 1) compared to chickens only primed with the Mass vaccine (group 2). Consistent with the results of respiratory signs, vaccinated chicken groups also showed a significant reduction of viral loads compared to unvaccinated/challenged birds (group 3) (Fig. 3.2). Similarly, no significant differences

($P < 0.05$) were detected in viral load in tears and tracheal samples between chickens vaccinated with Mass+ rLS/IBV.S2-4/91 (group 1) and Mass vaccine alone (group 2) (Fig. 3.2).

The antibody levels (S/P ratios) were not significantly different for the chickens vaccinated with Mass+ rLS/IBV.S2-4/91 (group 1) compared to vaccinated with Mass vaccine alone (group 2) at 16 and 26 DPC as well as before challenge (Fig. 3.3).

4. Discussion

Newcastle disease virus has been used extensively as a recombinant vector [146, 150, 151]. The advantages and disadvantages of this type of vector have been discussed in previous work and by others [122, 145, 146]. Confirming previous work [122], the insertion of the S2 gene of IBV between the P and M genes of NDV did not alter the biological properties (MDT and ICPI) of the lentogenic parental LaSota strain.

We have previously shown that S2 expressed from rLS effectively protects against Ark challenge when used in a prime and boost regime with Mass. Several studies have reported that the IBV 4/91 vaccine confers protection across IBV serotypes and has been designated as a "protectotype" by Europeans [141, 144, 152, 153]. Because the protection conferred by this vaccine strain cannot be explained by S1 homology, we considered the possibility that it might be conferred by S2, and developed rLS encoding S2 of IBV 4/91 (rLS IBV.S2-4/91) to evaluate its possible role in cross-protection. Despite the similarity of amino acid percent identity between S2 of 4/91 and Ark to the percent S2 amino acid identity between 4/91 and various challenge strains [142-144, 152], vaccination with 4/91 S2 produced no significant protection against Ark challenge

based on respiratory signs and viral load in tears and tracheas. Indeed, the partial level of protection conferred by the dissimilar Mass vaccine was not improved by the recombinant rLS IBVS2-4/91. The level of protection conferred by Mass was consistent with previous results obtained under experimental conditions [154]. Thus, protection observed in other studies cannot be attributed to similarity in S2 between 4/91 and heterotypic challenge strain. The S2 sequences of the Q1 and QX challenge strains actually used in the 4/91 cross-protection studies are not available and our conclusion was based on S2 sequences of Q1- and QX strains available in GenBank.

Unfortunately, after the initial careful work by Cook et al. [141] showing that IBV 4/91 induces cross-protection, most studies that followed using the vaccine strategy Mass vaccine on day 1 followed by booster vaccination with IB 4/91 lack relevant control groups [142-144, 152, 155]. The most common protocol used included Mass alone and Mass followed by 4/91, but Mass followed by Mass, 4/91 followed by 4/91, as well as single 4/91 vaccinations were frequently lacking in those investigations. Another relevant aspect absent in investigations regarding vaccines of serotype 793B are immunological studies including, for example, transference of T lymphocytes between vaccinated and naive chickens (of the same major histocompatibility complex haplotype) and cross-neutralization assays. Such would unequivocally demonstrate that 4/91 is indeed inducing heterotypic immunity. Another option for its cross protective capabilities could unfortunately be that this strain is simply extremely invasive and displaces wild regional strains providing apparent protection under those circumstances rather than real heterologous immunity. Initial evidence towards the idea that this strain maybe very invasive was provided by a group in Italy where they indicated that after eliminating 4/91

vaccination the problems of 4/91 type outbreaks were also eliminated [153]. Outbreaks of 4/91 type virus have been increasingly seen in different countries, for example in Egypt (Dr. A.M. Ghetas, personal communication), after the introduction of this vaccine.

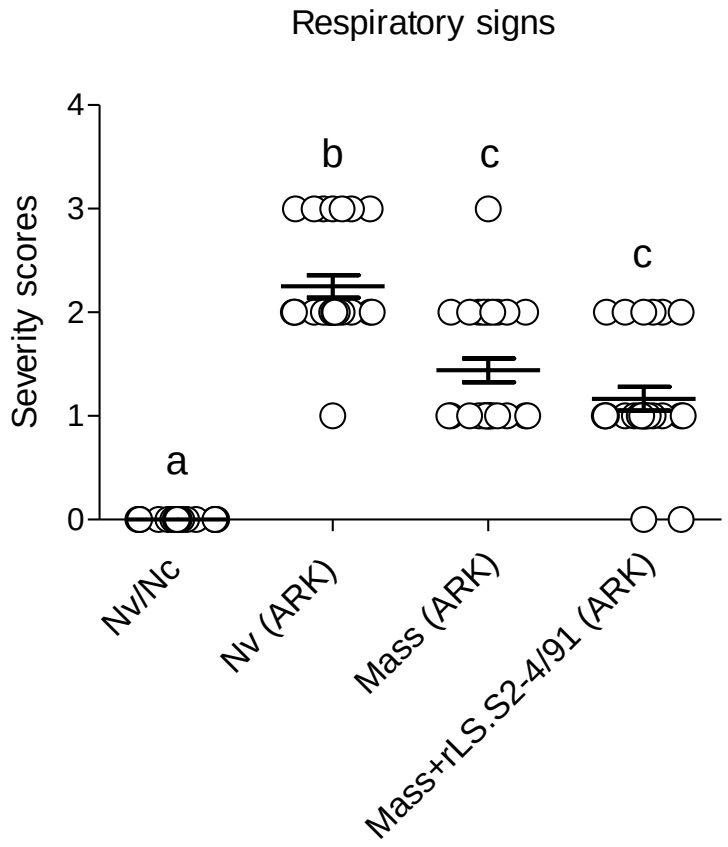
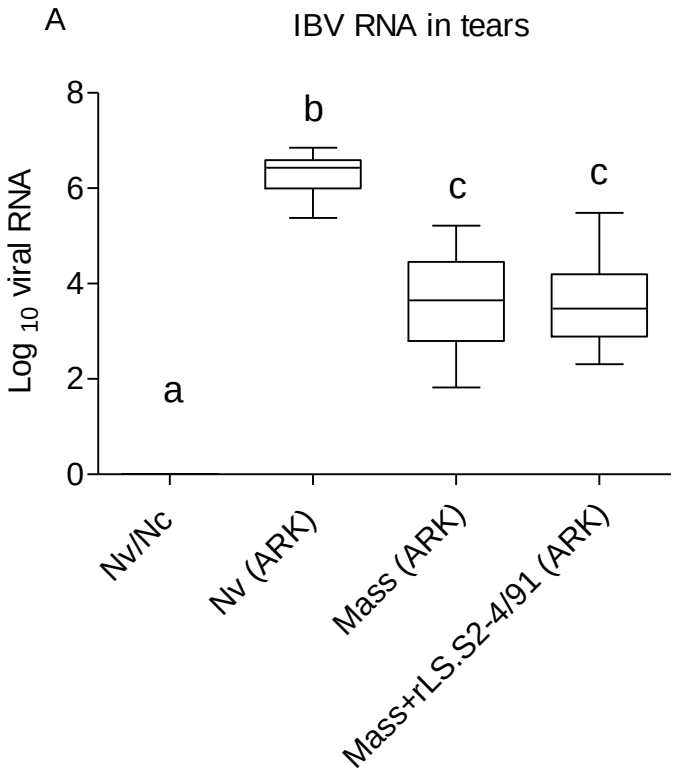


Fig. 3. 1. Respiratory signs after Ark challenge in chickens that were primed at day 1 of age with an attenuated Massachusetts (Mass)-type vaccine and boosted with rLS/IBV.S2-4/91 at 14 days of age (Mass+ rLS.S2-4/91), and chickens primed with Mass vaccine only (Mass). Severity of signs were scored (average ±SEM) 5 days after challenge. Nv/Nc =non-vaccinated/non-challenged and Nv (ARK) = non-vaccinated Ark challenged. Significant differences *($P < 0.05$)* indicated by different letters.


A
IBV RNA in tears
Log 10 viral RNA
8
6
4
2
0
a
b
c
c
Nv/Nc
Nv (ARK)
Mass (ARK)
Mass+rLS.S2-4/91 (ARK)

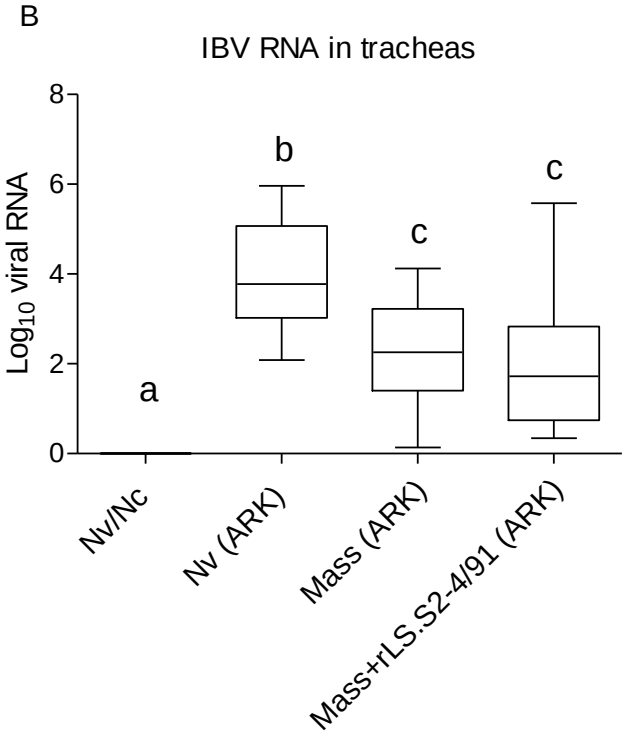

B
IBV RNA in tracheas
Log10 viral RNA
8
6
4
2
0
a
b
c
c
Nv/Nc
Nv (ARK)
Mass (ARK)
Mass+rLS.S2-4/91 (ARK)

Fig. 3. 2. Relative viral RNA levels in (A) tears and (B) tracheas in chickens that were primed at day 1 of age with an attenuated Massachusetts (Mass)-type vaccine and boosted with rLS/IBV.S2-4/91 at 14 days of age (Mass+ rLS.S2-4/91), and chickens primed with Mass vaccine only (Mass). Nv/Nc =non-vaccinated/non-challenged and Nv (ARK) = non-vaccinated Ark challenged. IBV RNA detected 5 days post- Ark challenge by Taqman qRT-PCR. Boxes: 25th percentile, median, 75th percentile; Whiskers: Min & Max. Significant differences ($P < 0.05$) indicated by different letters.

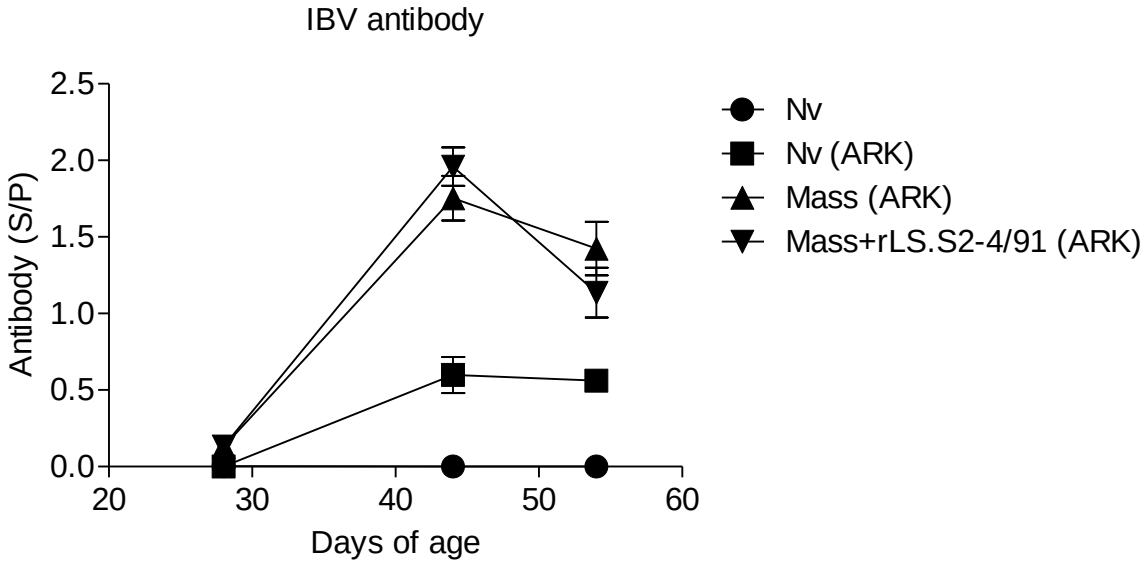


Fig. 3. 3. IBV antibodies in chicken sera pre-challenge (28 days of age), 16 days post-challenge (44 days of age) and 26 days post-challenge (54 days of age) detected by ELISA [sample/positive ratio (S/P)]. Both vaccinated groups showed significantly higher antibody levels than unvaccinated/challenged and unvaccinated non-challenged chickens

($P < 0.05$). No significant differences detected between Mass only and Mass+rLS.S2-4/91. Statistically significant differences not shown by letters for simplicity reasons.

Table 3.1. Pathogenicity of rLS/IBV.S2-4/91 virus determined by mean death time in embryonated eggs (MDT) and intracerebral pathogenicity index (ICPI) in day-old chickens. Titers achieved as measured in embryonated eggs (EID_{50}), cell culture ($TCID_{50}$), and by hemagglutinating activity (HA).

Virus	MDT	ICPI	EID_{50}	$TCID_{50}$	HA Titer
rLaSota	110 h	0.15	ND	ND	ND
rLS/IBV.S2-4/91	>144 h	0.19	$10^{8.5}$	$10^{8.3}$	2^{11}

CHAPTER 4

Role of Spike Protein S2 Domain in Attachment and Selection of ArkDPI IBV Vaccine Subpopulations in Chickens

Abstract

Infectious bronchitis virus (IBV) is an economically important coronavirus of chickens. IBV undergoes an evolutionary process by point mutations, recombination, and selection that results in continuous emergence of new serotypes. Live attenuated ArkDPI-derived vaccines contribute to emergence of new IBV variants in vaccinated chickens by natural selection, resulting in vaccine virus persistence and increased vaccine virus virulence. Previously, five minor vaccine virus subpopulations selected in chickens, designated component (C) (C1-C5), were identified, based on S1 gene sequences. The spike (S) protein of IBV contains a receptor-binding S1 subunit and a membrane-fusion S2 subunit. To examine the role of attachment in selection of vaccine subpopulations in chickens, we compared the binding efficiency of recombinant S1 and S-ectodomain proteins representing C2 (strongly selected) and C3 (weakly selected) ArkDPI-derived vaccine subpopulations with those representing the major vaccine population (negatively selected) to different chicken tissues. We hypothesized that spike proteins of vaccine virus subpopulations that are positively selected in chickens bind more efficiently to chicken tissues than that of the negatively selected vaccine major population. Secreted, trimeric, strep-tagged recombinant spike proteins representing S1 and S-ectodomains of C2 and C3 vaccine subpopulations and the vaccine major population were produced in mammalian cells from

codon-optimized constructs. Protein histochemistry of purified proteins confirmed that C2S1 bound more strongly than vaccine S1 to most chicken tissues tested. No binding to kidney or lung tissue by either S1 protein was detected. Unexpectedly, C3S1 binding was not detected to any of the tissues. Based on our previous observation that the S2 ectodomain of the C2 S protein greatly increased the binding of C2S1 to chicken tissues, we hypothesized that the S2 ectodomain of the weakly selected C3 vaccine subpopulation facilitated the binding of C3 spike protein to chicken tissues and thus contributed to selection of this vaccine subpopulation in chickens. As expected, C2 and vaccine major population S-ectodomain proteins bound chicken tissues more strongly than the corresponding S1 proteins, and bound to lung and kidney tissues. Furthermore, the C3 S-ectodomain bound to all tissues tested, confirming a role for the S2 ectodomain in tissue binding, and suggesting a role in virus attachment. However, in the context of the entire S ectodomain, consistent differences in binding among the vaccine subpopulations positively and negatively selected in chickens were not demonstrated, thus failing to support our hypothesis that differences in the S2 domains in selected vaccine subpopulations contribute to their selection in chickens by improving attachment.

1. Introduction

The avian coronavirus infectious bronchitis virus (IBV) is one of the most economically important viruses in the poultry industry worldwide. IBV shows extensive genotypic and phenotypic variability that is generated by mutation, recombination, and selection and results in continuous emergence of new serotypes [1, 83]. IBV Arkansas-type viruses (including Ark99, ArkDPI, and Arkansas-like viruses) are the most commonly isolated IBV in commercial broiler flocks in the southeastern United States [88, 89, 156, 157] despite extensive use of Arkansas

(Ark) serotype vaccines. Live attenuated Arkansas-Delmarva Poultry Industry (ArkDPI)-derived vaccines contribute to emergence of new IBV variants in vaccinated chickens by natural selection, via vaccine virus persistence, increased vaccine virus virulence, and selection of variant challenge viruses [37, 82, 83, 89, 95, 158]. We previously identified five minor vaccine virus subpopulations rapidly selected in chickens from ArkDPI-derived IBV vaccines, designated components (C) 1-5 [81, 83]. The selection of these viral subpopulations within 3 days post-vaccination suggests they replicate better in chickens than the major vaccine population [81, 83, 128]. Indeed, the selected vaccine subpopulations reach higher viral loads in tears of vaccinated chickens than the major vaccine population [128].

The envelope-anchored spike (S) protein of IBV mediates viral entry into host cells [3, 56]. The S1 subunit mediates viral attachment to host cells and induces virus-neutralizing antibodies [45, 107, 126]. In addition, S1 protein differences affect the species and tissue tropism of IBV [50, 52, 58, 77, 80, 159] and several other coronaviruses [75, 79, 160, 161]. The S1 subunit shows extensive amino acid sequence variability among IBV strains, which leads to the virus's immunological escape [1, 91], while the S2 subunit, which is responsible for membrane fusion, is more conserved among IBV strains [121, 122, 125].

Protein histochemistry of recombinant S1 proteins of three IBV Massachusetts serotype strains, virulent M41, H120 vaccine strain, and the avirulent mammalian-cell-culture-adapted Beaudette strain, showed that binding to chicken trachea in vitro correlates with the pathogenicity of those strains in vivo [52]. Although Beaudette S1 protein alone was not sufficient for binding to chicken trachea [52], chorioallantoic membrane (CAM), or BHK21 cells, extension of S1 with the S2 part of the spike ectodomain allowed binding to BHK21 cells and CAM, in which Beaudette is known to replicate [42]. In addition, the spike ectodomain of

M41 strain showed increased attachment compared to M41 S1 protein to both CAM and chicken trachea [42, 58]. It has been demonstrated that S2 does not contain an additional binding site, but may contribute to the S1 avidity [42]. Together, these findings suggest that S1 and S2 might work together to determine the specificity of virus attachment [42, 58].

Previous work aimed at extending the principle that binding of recombinant S1 proteins to chicken tissues reflected the ability of IBVs to replicate in chickens to Ark serotype viruses, and demonstrating a role for improved attachment in selection of ArkDPI vaccine subpopulations in chickens, showed that recombinant S1 protein representing vaccine subpopulation C2 (strongly selected in chickens) bound chicken tissues better than recombinant S1 protein representing the negatively selected vaccine major population [162]. However, contrary to expectation, recombinant S1 protein representing subpopulation C3 (weakly selected in chickens) did not show detectable binding to chicken tissues. In the present work we evaluate the binding to different chicken tissues of S1 proteins of ArkDPI vaccine subpopulations C2 and C3 and the vaccine major population to confirm those results. In addition, we assess the role of the S2 ectodomain in attachment and selection of these subpopulations, especially C3, by comparison of binding to chicken tissues of entire S-ectodomains representing these subpopulations and the vaccine major population.

2. Materials and methods

2.1. Genes and expression vectors

Expression constructs to produce recombinant S proteins representing IBV ArkDPI vaccine subpopulations C2 and C3 and the vaccine major population were generated. The C2 subpopulation was chosen because it was positively selected in chickens, matches the S1

consensus sequence of ArkDPI vaccine subpopulations selected in chickens, and displays high S1 amino acid sequence similarity with the virulent ArkDPI strain field isolate (Genbank accession number AAB61013). The C3 subpopulation was weakly selected from ArkDPI-derived vaccines and matches the Ark-type field consensus at crucial positions.

Human codon-optimized sequences encoding C2, C3, or vaccine major population S1 [amino acids (AA) 19-538] were synthesized (GeneArt, Regensburg, Germany) and cloned into the pCD5 vector to generate recombinant S1 protein. Human-codon optimized sequences encoding the S2 ectodomains (S AA 544-1097) of C3 and vaccine major populations were also synthesized by GeneArt. Human codon-optimized sequences encoding the C2 S2-ectodomain were generated by site-directed mutagenesis (splice overlap extension PCR) from C3 S2-ectodomain coding sequences as described [163, 164]. Specific mutations in three codons (P690S, L1036F, and G1067D) were introduced sequentially by incorporating nucleotide changes into the overlapping oligonucleotide primers. The reaction required two separate amplifications, two flanking primers complementary to the ends of the target sequence, and two internal primers that contain the desired mutation (Table 4.1). Then, the DNA fragments were combined in a subsequent fusion reaction in which the overlapping ends annealed to form full-length double-stranded mutant S2 coding sequences. The full-length mutant S2 coding sequences were amplified further by PCR. *KpnI* and *PacI* were used for restriction digestion for cloning the S2-ectodomain-encoding sequences into the pCD5 vector already encoding the S1 domain to produce S-ectodomain protein as described [42]. At the S1/S2 border, the furin cleavage site sequence RRSRR was replaced by GGGVP to avoid cleavage of the full length S-ectodomain [42]. All clones were sequenced to confirm the desired mutations, and absence of any other mutations. The S1- and S-ectodomain-coding sequences were flanked by sequences encoding an

N-terminal CD5 signal sequence and sequences encoding a C-terminal artificial GCN4 trimerization motif and Strep-tag II for purification and detection of proteins, as described [52].

Expression constructs for recombinant S-ectodomain of C3 and vaccine major (V) S1s extended with M41 S2 were generated. Human-codon optimized sequences encoding the S2 ectodomain of M41 were cloned into the pCD5 vector already containing the C3 or V-S1 domain, using *KpnI* and *PacI* restriction enzymes to produce C3S1-M41S2 and VS1-M41S2. All clones produced were confirmed by sequencing.

2.2. Cells

Human embryonic kidney (HEK) 293T cells were maintained in Dulbecco's modified Eagle's medium (BioWhittaker, Walkersville MD) supplemented with 2% glutamine, 10% fetal bovine serum (Seradigm, Providence, UT) and 0.1 mg/ml gentamicin (Amresco, Solon, OH).

2.3. Recombinant protein production and purification

Soluble trimeric secreted recombinant S1 and S-ectodomain proteins of C2, C3 and vaccine major population (V) were produced in human embryonic kidney (HEK) 293T cells as described [42, 52, 129]. In brief, the expression vectors encoding S1 or S-ectodomain were transfected into HEK293T cells. The tissue culture supernatants were harvested at 6 days post-transfection. Recombinant protein expression was analyzed by western blotting using horseradish peroxidase (HRPO)-conjugated Strep-Tactin (IBA GmbH, Göttingen, Germany). Recombinant proteins were purified using Strep-Tactin® Sepharose columns according to the manufacturer's instructions (IBA GmbH, Göttingen, Germany). The concentration of purified proteins was initially determined by Qubit® 2.0 fluorometer (Invitrogen, Carlsbad, CA). The purified proteins

were confirmed and concentrations normalized by electrophoresis in Mini-PROTEAN®TGX Stain-Free™ Precast Gels (Bio-Rad, Hercules, CA) under denaturing conditions.

2.4. Protein histochemistry

The binding efficiency of C2, C3 and V S1 and S-ectodomain proteins to tissue sections, prepared from healthy specific pathogen free (SPF) 40-60 day old white leghorn chickens, was assessed by protein histochemistry as described [42, 129] with some modifications: antigen retrieval was conducted at 80° C for 30 min, Tris buffers were substituted for phosphate buffers, slides were blocked with universal negative serum (Biocare, Pacheco, CA) instead of 10% goat serum, and the addition of most reagents and washing steps were performed by an intelliPATH FLX automated slide stainer (Biocare, Pacheco, CA). S proteins and 3-amino-9-ethyl-carbazole (AEC+; Dako, Carpinteria, CA) were added manually. Briefly, S proteins (100 µg/ml for S1 and 50 µg/ml for S-ectodomain) pre-complexed with Strep-Tactin-HRP (IBA GmbH, Göttingen, Germany) were incubated with deparaffinized and rehydrated tissue sections overnight at 4 °C. Bound S protein was visualized with AEC+ chromogenic substrate. The tissues were counterstained with hematoxylin and mounted with Lerner AquaMount (Covance, Princeton, NJ). Images were captured using Aperio eSlide Manager-Digital Pathology Software.

3. Results

Codon-optimized sequences encoding S1 and S-ectodomain proteins of IBV ArkDPI vaccine subpopulations C2 and C3 selected in chickens and the vaccine major population were cloned into the pCD5 expression vector preceded by sequences encoding the CD5 signal peptide and followed by sequences encoding the GCN4 trimerization domain and Strep-tag II. The C2 S2-

coding sequences for construction of the C2 ectodomain expression construct were successfully generated by site-directed mutagenesis of C3 S2 domain-coding sequences and confirmed by sequencing. The generated clones were transfected into HEK293T cells, and the secreted S1 and S-ectodomain proteins were purified. As seen in Fig. 4.1, all S1 and S-ectodomain proteins representing the vaccine subpopulations C2 and C3 and V were detected by electrophoresis. In addition, the proteins produced were highly glycosylated, as S1 migrated to 105 kDa and S-ectodomains to 200 kDa as previously demonstrated [42, 52].

Comparing the binding activity of S1 subunits of C2, C3 and vaccine major population to different chicken tissues, C2S1 bound more strongly than vaccine S1 to all tissues tested including: tracheal mucosa, conjunctival epithelium, nasal mucosal glands and epithelium, and the epithelium of the choana, cecal tonsils, and cloaca (Fig. 4.2). C3S1 did not bind detectably to any of the tissues. None of the S1 domains bound to kidney or lung tissues (not-shown).

The S-ectodomain, comprised of the S1 domain extended with the S2 ectodomain, of both the C2 and C3 subpopulations as well as the vaccine major population showed strong binding affinity for all tissues tested (Fig. 4.3). Moreover, S ectodomains bound to lung and kidney tissues (Fig. 4.3) indicating that S2 is required for binding to these tissues. Addition of S2 also allowed the C3 spike protein to bind to most tissues similarly to the C2 ectodomain. Thus, S2 increases the binding efficiency of C2 and vaccine major population spike proteins and is essential for binding of C3 spike to chicken tissues. However, in this and several other protein histochemistry experiments, consistent differences in binding affinity among the S-ectodomains of the strongly selected C2, weakly selected C3, and negatively selected vaccine major

population were not demonstrated. Thus, our hypothesis that differences in the C3 S2 ectodomain contribute to selection of the C3 vaccine subpopulation in chickens was not confirmed.

To further investigate the role of differences in S1 in binding efficiency, attachment, and ultimately selection of the C3 subpopulation in chickens, we compared binding of chimeric constructs of C3 and V S1 extended with the S2 of M41 strain. As was the case for C3 and V S-ectodomains, extension of C3S1 or VS1 with the S2 ectodomain of the M41 strain increased the tissue binding of V and allowed the binding of C3 in all tissues tested (Fig. 4.4). Combined with the same S2 ectodomain, C3 S1 appeared slightly inferior to V S1 in binding to most tissues.

4. Discussion

In this study, we compared the binding patterns of spike S1 and S-ectodomain protein of ArkDPI vaccine subpopulations strongly or weakly positively selected in chickens to the vaccine major population negatively selected in chickens [81, 83]. Protein histochemistry of the S1 subunit of vaccine subpopulations and the vaccine major population showed that S1 of the strongly selected C2 subpopulation bound more strongly than that of the vaccine major population to most tissues tested, while S1 of the weakly selected C3S1 did not bind to most tissues. None of the tested S1 subunits bound to kidney and lung tissues. These results are in partial agreement with previous studies on Mass serotype IBV strains that showed that the S1 subunit of the spike protein of the virulent IBV strain M41 was sufficient for attachment to chicken respiratory tract and kidney tissues, and that it bound better than S1 protein of the attenuated H120 vaccine strain, while the S1 protein of the highly-attenuated Beaudette strain

was not sufficient for binding to chicken trachea [52], embryonic CAM tissue and mammalian cell lines [42, 58]. This was explained by accumulating mutations in the spike S1 gene as a result of adaptation of IBV Beaudette strain to Vero cells and thus, the sequence differences contribute to host range [42]. The S1 amino acid differences between C2S1 and C3S1 are at four AA positions that may be critical for binding and attachment. Our results differ from those obtained with M41 S1 protein in that the S1 subunit of ArkDPI vaccine subpopulations is not sufficient for binding to kidney and lung tissues; however the S-ectodomain binds efficiently. We have extended the results obtained using Mass serotype S1 domains by showing that S1 of the positively selected C2 ArkDPI vaccine subpopulation is able to more strongly bind to numerous relevant chicken tissues than the S1 of weakly selected C3 subpopulation and vaccine major population.

Interestingly, our results showed that all tested S-ectodomains bound to all chicken tissues tested, indicating that S2 is required to improve the binding efficiency to chicken tissues. Similar results were demonstrated with M41 strain binding to chicken trachea [58]. Addition of the S2 ectodomain allowed the C3 spike ectodomain protein to bind similarly to the C2 and vaccine S-ectodomains in some experiments. However, these results were not consistent; in some experiments C2 ectodomain bound better than C3 and V ectodomains. Using chimeric S-ectodomain proteins, Promunktod et al. concluded that S2 does not contain an additional independent receptor binding site that would explain its contribution to the affinity of S for receptors [42]. Binding was explained by the combined effect of the S1 and the S2 subunits of the spike ectodomain that were essential for attachment. Our possible explanation for improved attachment with S-ectodomains compared to S1, is that the S2 subunit is necessary for the S1 protein to adopt a conformation optimal for binding. Structures of trimeric S-ectodomains of

other coronaviruses determined by cryo-electron microscopy suggest that the trimeric structure is important for the conformation of S1, because the S1 domains of the monomers are interwoven in the trimer [44, 132]. In the recombinant S1 protein used in this study, the artificial trimerization domain immediately follows the S1 domain and could thus artificially constrain the trimeric S1 in a suboptimal conformation. When the S2 ectodomain is included between S1 and the trimerization domain, the trimers might be closer to their normal conformation.

Promunktod, et al. [42], found that although the extension of the Beaudette S1 protein with the Beaudette S2 ectodomain allowed weak binding to chicken trachea, a chimeric protein consisting of the Beaudette S1 subunit and M41 S2 ectodomain was not able to bind, suggesting that S1 and S2 domains from different strains were not able to cooperate effectively. In contrast, our results using a chimeric S ectodomain with Ark-derived S1 subunits and the M41 S2 ectodomain showed that even S1 and S2 domains from different serotypes are able to cooperate to bind chicken tissues with high affinity.

In conclusion, the spike S2 domain may have an important role in attachment especially of the weakly selected C3 subpopulation. However, in contrast to a possible role in selection for differences among vaccine subpopulations in the ability of their S1 subunits to bind to cells, we were unable to clearly demonstrate a role for differences in their S2 subunits in subpopulation selection.

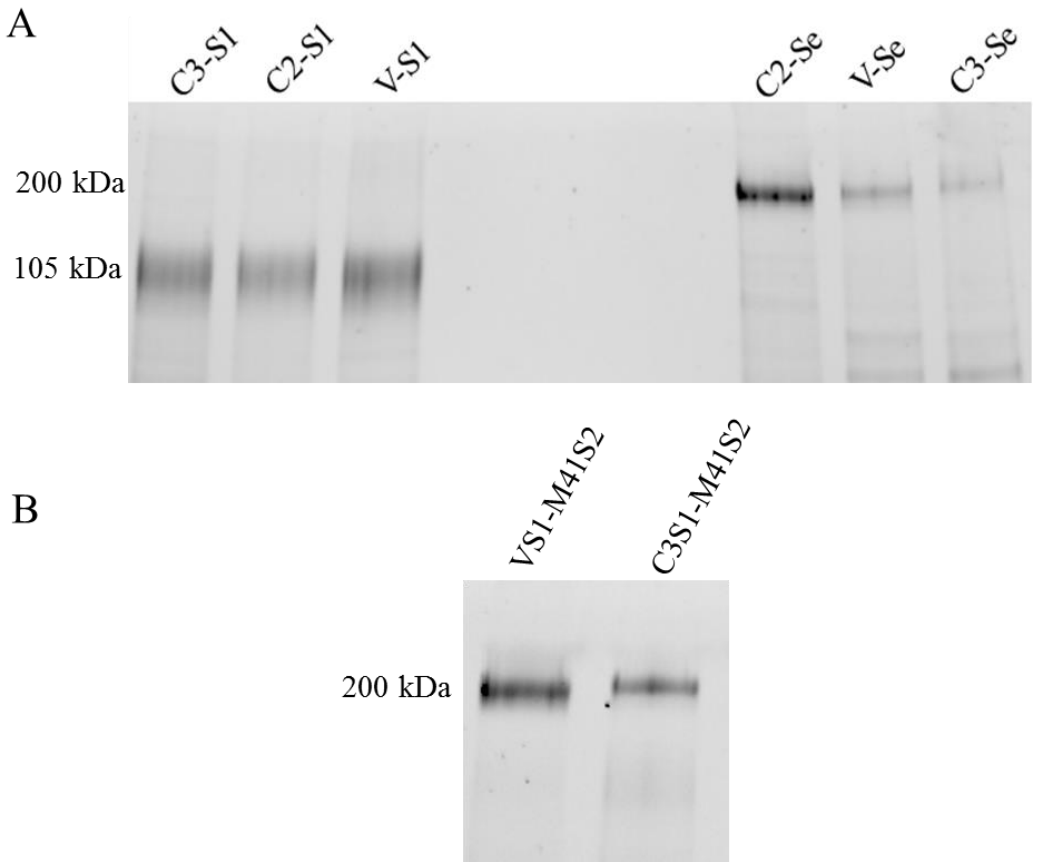


Fig. 4.1. Purified recombinant IBV S1 and S-ectodomain (Se) proteins expressed in HEK293T cells. The expression and purification of recombinant S1 and S-ectodomain proteins representing C2 and C3 ArkDPI vaccine subpopulations and vaccine major population (V) (A) as well as all generated chimera recombinant proteins (B) were confirmed and concentrations normalized by electrophoresis in Mini-PROTEAN®TGX Stain-Free™ Precast Gels. The glycosylated S1 proteins are approximately 105 kDa and S ectodomains are approximately 200 kDa.

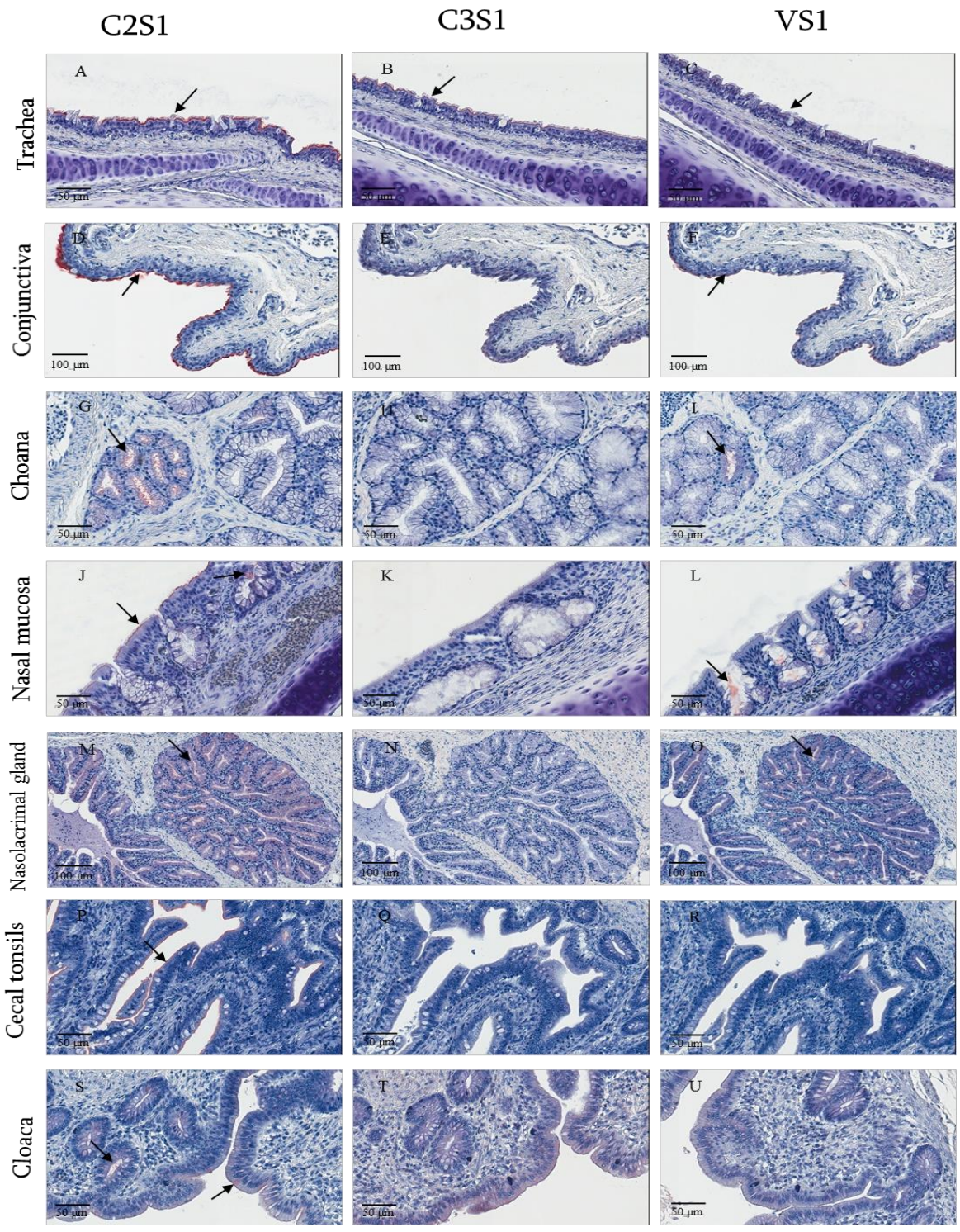
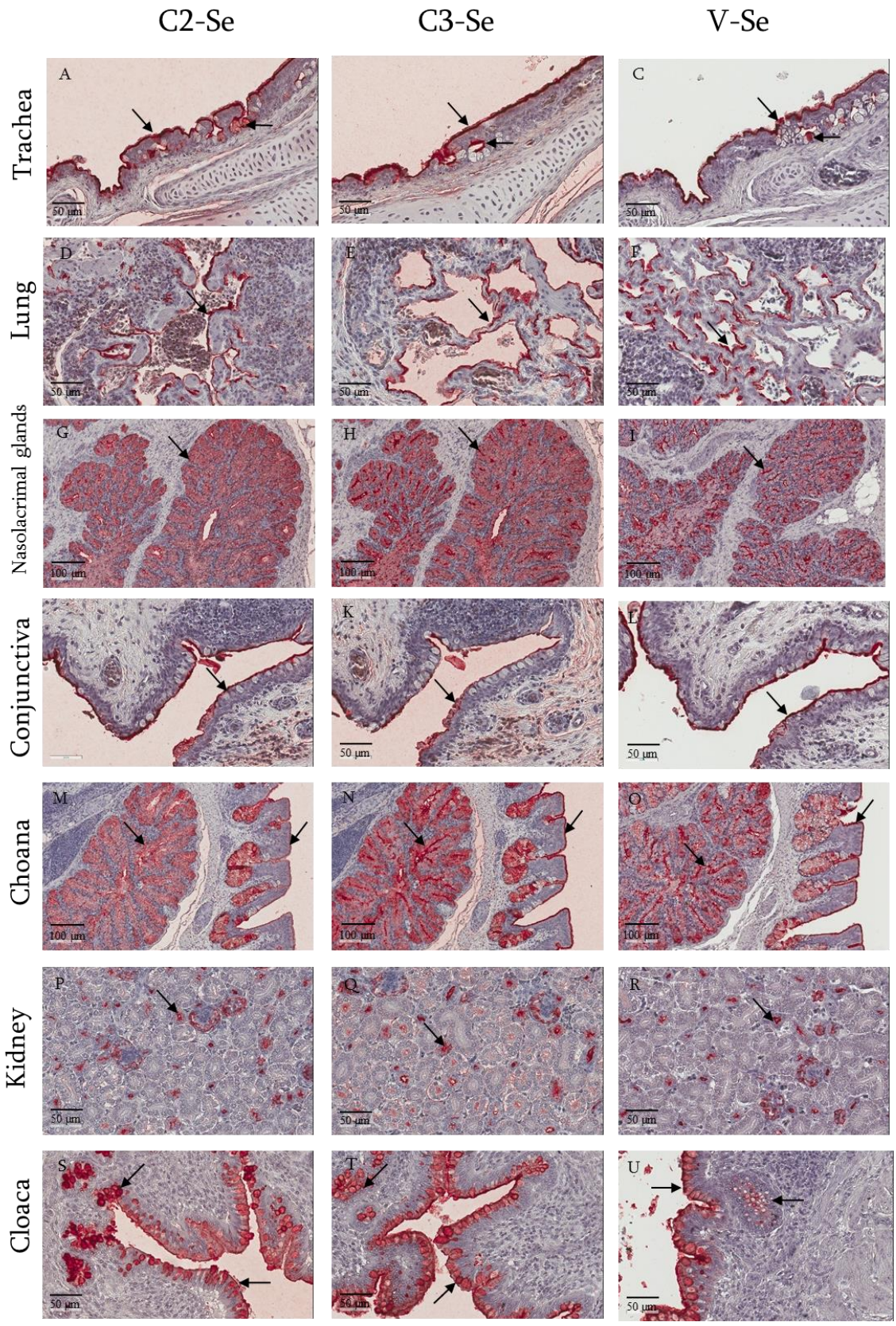


Fig. 4.2. Protein histochemistry demonstrating recombinant IBV Ark-type S1 binding in various chicken tissues. AEC+ chromogenic substrate was used to identify bound spike protein as indicated by red staining (arrows). S1 protein of C2 (A) showed stronger binding

to cilia and tracheal epithelium compared to vaccine major population (V) (C). C2S1 protein also showed more extensive binding to conjunctival epithelium (D) and to epithelial apical surfaces and secretory product in the choanal glands (G) and nasolacrimal glands (M) than did VS1 protein (F, I, O). C2S1 showed binding to apical surface of the nasal mucosal epithelium and mucus glands (J) while VS1 showed binding only to mucus glands (L). C2S1 showed binding to apical surface of the cecal tonsil intestinal epithelium (P), while binding by VS1 was not detected (R). C3S1 did not show readily discernable binding to any of the tissues tested (B, E, H, K, N, Q).


C2-Se
C3-Se
V-Se
Trachea
Lung
Nasolacrimal glands
Conjunctiva
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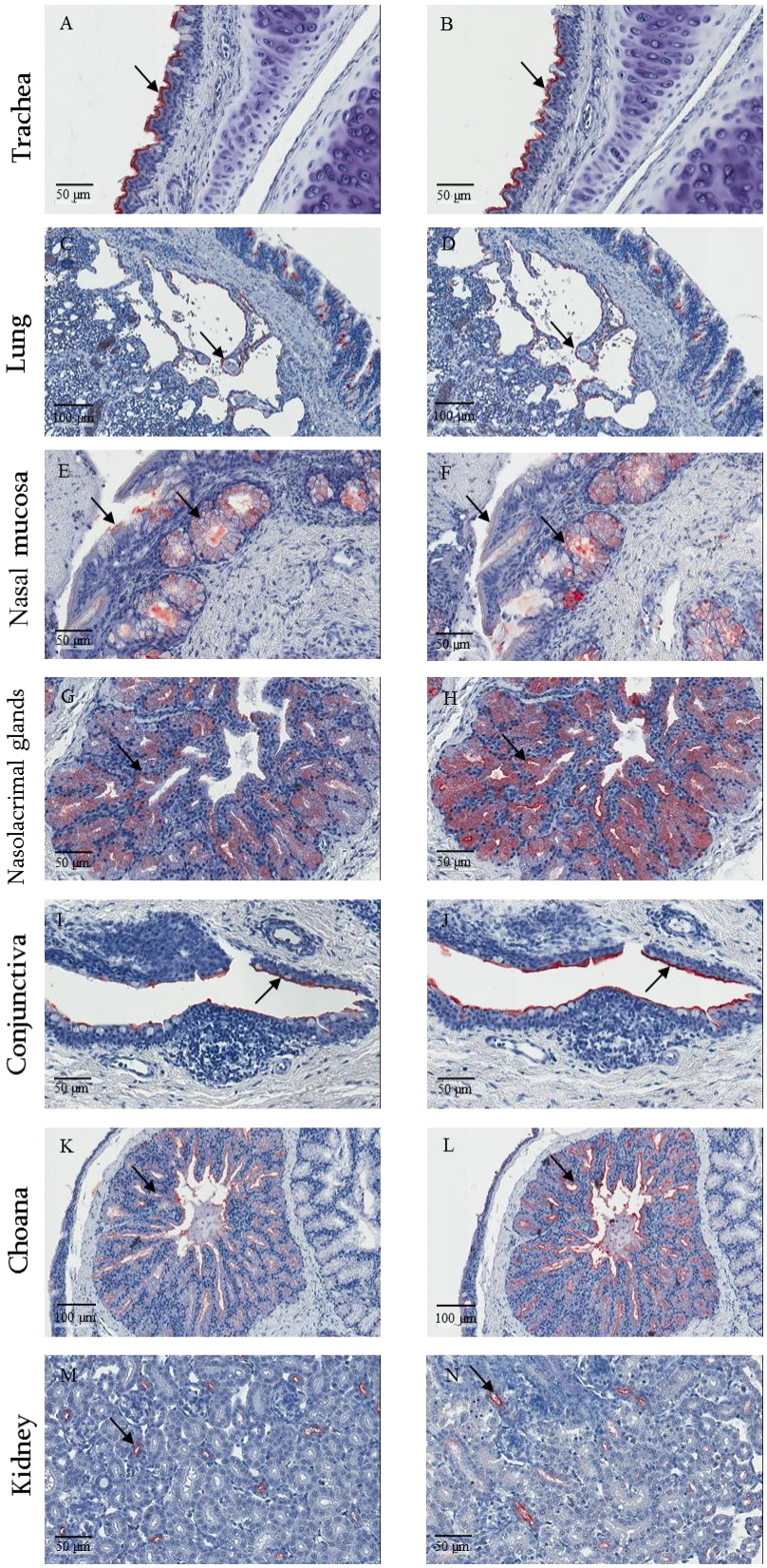
N
O
P
Q
R
S
T
U
50 µm
100 µm

Fig. 4.3. Protein histochemistry of recombinant IBV S-ectodomain (Se) proteins of C2, C3 ArkDPI derived vaccine subpopulations as well as vaccine major population (V) in different chicken tissues. Bound spike protein was identified by AEC+ chromogenic substrate as indicated by red staining (arrows). Se proteins of C2 (A), C3 (B) and V (C) bound similarly to the cilia, epithelium, and the mucin-containing goblet cell secretory vesicles of the trachea. C2, C3, and V-Se showed similar binding to epithelium lining the pulmonary parabronchi (D, E, F). C2 (G), C3 (H), and V (I) Se proteins showed similar binding to nasolacrimal glandular epithelium. All three proteins bound similarly to the conjunctival epithelium (J, K, L), the choanal submucosal glandular epithelium (M, N, O), the epithelial apex of scattered renal tubules (P, Q, R), and also the cloacal epithelium (S, T, U).


C3S1-M41 S2
VS1-M41 S2
Trachea
Lung
Nasal mucosa
Nasolacrimal glands
Conjunctiva
Choana
Kidney
A
B
C
D
E
F
G
H
I
J
K
L
M
N
50 µm
100 µm

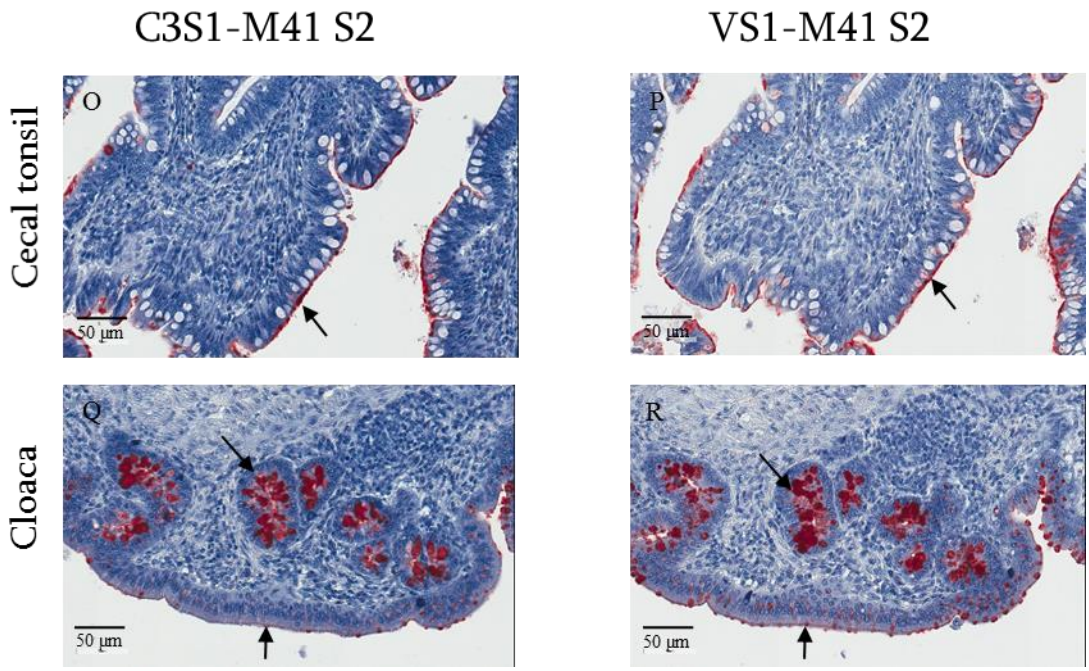


Fig. 4.4. Protein histochemistry of recombinant IBV chimeric spike ectodomain proteins in different chicken tissues. Recombinant S1 of C3 ArkDPI vaccine subpopulation or the vaccine population (V) were extended with S2 domain of IBV Massachusetts M41 strain and expressed in mammalian cells. Bound spike protein identified by AEC+ chromogenic substrate and indicated by red staining (arrows). C3S1-M41S2 protein showed strong binding to the cilia and tracheal epithelium (A) compared to VS1-M41S2 (B). Both C3S1-M41S2 and VS1-M41S2 showed similar binding to epithelium lining the pulmonary parabronchi (C, D) and the nasal mucosal epithelium and mucus glands (E, F), respectively. VS1-M41S2 showed strong binding affinity to nasolacrimal glandular epithelium (H), to conjunctival epithelium (J), and to the choanal submucosal glandular epithelium (L) compared to C3S1-M41S2 (G, I, K), respectively. Both C3S1-M41S2 and VS1-M41S2 showed binding to epithelial apex of scattered renal tubules (M, N), to cecal tonsil epithelium (O, P), and to cloacal glands and epithelium (Q, R).

Table 4.1. Primers used in site-directed mutagenesis. Underlining indicates the introduced mutations. Primers VBS1syn-2F and pCD5R are the primers complementary to the ends of the sequence to be amplified.

Primer	Sequence (5' to 3')
VBS1syn-2F	AAAGTGAACCCCTGCGAG
pCD5R	TCCTCGATCTGCTTCATGC
C3S2-P690S-F	GCTGACCAGCCCCAGCAG
C3S2-P690S-R	CTGGGGCTGGTCAGCAGCAGG
C3S2-L1036F-F	CGACTTCGACTTCGACGACGAGC
C3S2-L1036F-R	CGTCGAAGTCGAAGTCGTCGTTG
C3S2-G1067D-F	CTGGACATCGACAGCGAGATCG
C3S2-G1067D-R	CGATCTCGCTGTCGATGTCCAG

CHAPTER 5

Characterization of Receptor-binding Domain of Ark-type Infectious Bronchitis Virus S1 Protein

Abstract

Infectious bronchitis virus (IBV) infection is initiated by the binding of spike (S) protein to the host cell. The S1 domain/subunit of S contains the receptor-binding domain (RBD) for all coronaviruses for which an RBD has been identified, while the S2 domain/subunit functions in viral membrane fusion. The RBD of Massachusetts serotype IBV strain M41 has been mapped to the N-terminal domain of the S1 subunit. To map the RBD of the S1 protein of an IBV Arkansas (Ark) serotype strain, secreted recombinant trimeric strep-tagged full-length and truncated S1 proteins of ArkDPI-derived vaccine subpopulations selected in chickens (C2 and C3) as well as the vaccine (V) major population were expressed in mammalian cells. The binding affinities of purified proteins to different relevant chicken tissues were analyzed. Protein histochemistry showed that the N-terminal 19-258 amino acids (NTD_{258}) of the S1 of vaccine subpopulation C2 are necessary and sufficient for binding to all chicken tissues tested except lung tissue. Additionally, the binding efficiency of NTD_{258} protein is greater than full-length S1 protein, suggesting that the protein folding structure of full-length S1 protein might partially mask binding sites. Comparing the binding activity of NTD_{258} protein to S-ectodomain protein, the entire S-ectodomain showed improved binding to several chicken tissues and was required for

binding to lung tissue. The NTD_{258} protein of ArkDPI-derived vaccine subpopulation C3 as well as vaccine major population also bound to tested chicken tissues better than their complete S1 proteins. The NTD_{258} of C2 showed improved binding compared to those of the C3 and vaccine major population. NTD_{258} of the vaccine major population with amino acid changes to match selected vaccine subpopulation C2 (Y43H or S213A) showed improved binding efficiency. Together, our results indicate that the RBD of IBV Ark strain is located within the N-terminal 19-258 amino acids of the S1 protein and the amino acids at positions 43 and 213 are critical for binding affinity.

1. Introduction

The avian coronavirus infectious bronchitis virus (IBV) is one of the most economically important viruses in the poultry industry worldwide. IBV shows genetic diversity generated by mutation, recombination, and selection, which results in continuous emergence of new serotypes that prevents effective vaccination [1]. Live attenuated vaccines participate in evolutionary processes in vaccinated chickens, resulting in increased vaccine virus virulence and persistence [83, 95]. We previously identified five minor vaccine virus subpopulations selected in chickens from Arkansas-Delmarva Poultry Industry (ArkDPI)-derived IBV vaccines, designated components (C) 1-5 [81, 83]. The selection of these viral subpopulations within 3 days post-vaccination suggests they replicate better in chickens than the major vaccine population [81, 83].

The envelope-anchored trimeric spike (S) protein of IBV mediates viral entry into host cells [3, 56]. S protein contains an ectodomain, a transmembrane anchor, and a short intracellular tail. The ectodomain consists of a receptor-binding S1 subunit and a membrane-fusion S2 subunit. The major genetic variations of IBV have been attributed to the S1 subunit [1, 91, 92], while the

S2 sequences are more conserved [122]. In addition, the S1 subunit determines the serotype of IBV and is the main target of neutralizing antibodies [45, 107, 126]. Moreover, S1 protein differences affect the species and tissue tropism of IBV [52, 58, 80] and several other coronaviruses such as porcine transmissible gastroenteritis virus [75, 160], and sudden acute respiratory syndrome (SARS) coronavirus [79]. While S2 does not contain an additional binding site, it increases the avidity of S1 and thus likely contributes to viral attachment [42, 165].

Receptor binding domains (RBDs) are the most important target of neutralizing antibodies, preventing virus-receptor interaction and thus may work as subunit vaccines against infection [57, 105, 106, 166]. Two independent RBDs that are located in either the N-terminal domain (NTD) or C-terminal domain (CTD) of the S1 domain of the spike protein have been identified for a wide variety of coronaviruses [56, 105, 106, 166]. The RBD of the IBV Massachusetts strain (M41) has been recently mapped to the S1 NTD [50]. Promkuntod, et al. [50], demonstrated that the NTD of M41 S1 (AA 19–272), but not the CTD (AA 273-532), is required and sufficient for binding to the chicken respiratory tract in an α-2,3-sialic acid-dependent manner. In addition, the NTD protein has a similar binding affinity compared to the full-length M41 S1 protein [50]. They also identified four specific amino acids within the N-terminal residues 19–69 that were essential for binding [50].

The RBD of the IBV Arkansas DPI- (ArkDPI) genotype, the most frequently isolated IBV genotype in the southeastern United States [88, 156], has not yet been mapped or identified. Therefore, in this study we generated truncated S1 proteins to identify the RBD of the S1 domain of an IBV ArkDPI strain. The binding efficiency of different S1 domains to relevant different chicken tissues was determined and compared to full-length S1 and S-ectodomain proteins. In

addition, the binding affinities of S1 subdomains of two ArkDPI-derived vaccine viral subpopulations selected in chickens and the vaccine major population [81, 83] were assessed.

2. Materials and methods

2.1. Construction of expression plasmids

The sequence of the S gene representing the IBV ArkDPI vaccine subpopulation previously designated C2 (GenBank accession ABY66333) was used to produce recombinant C2-NTD and CTD. Human codon-optimized sequence encoding C2S1 [amino acids (AA) 19-538] was synthesized (GeneArt, Regensburg, Germany) and cloned into the pCD5 vector. To generate S1 NTD and CTD subdomains, the predicted folding was first determined using FoldIndex (https://fold.weizmann.ac.il/fldbin/findex)[167]. However, no clear unfolded region separating the putative NTD and CTD, as had been found for the M41 S1 protein [50], was identified (Fig. 5.1A). Therefore, we relied on a ClustalW alignment (not shown) of the S1 AA sequences of M41 and C2 to identify putative C2 S1 NTD and CTD analogous to those identified for M41 S1. Constructs coding for C2S1-NTD_{258} (AA 19-258), NTD_{137} (AA 19-137), CTD_{259} (AA 259-538), CTD_{258} (AA 258-538) and CTD_{138} (AA 138-538) were produced from the codon-optimized C2 S1 expression construct using the PCR primer sets shown in Table 5.1. Restriction sites *Nhe*I and *Kpn*I were used to clone these S1 subdomains into the pCD5 vector and the sequences were verified by nucleotide sequencing. The S1 and subdomain-coding sequences were flanked by sequences encoding an N-terminal CD5 signal sequence and sequences encoding a C-terminal artificial GCN4 trimerization motif and Strep-tag II for purification and detection of proteins, as described [42]. Similarly, NTD_{258} (AA 19-258) subdomains coding for ArkDPI derived vaccine viral subpopulations previously designated component C3 (C3-NTD_{258}) and the vaccine major

population (V) (V NTD_{258}) [81, 83] were generated. To further investigate the role in attachment of two AA differences between NTD_{258} of vaccine subpopulations selected in chickens and V NTD_{258} [81, 83], human codon-optimized expression constructs encoding the vaccine major population NTD_{258} with amino acid changes Y43H or S213A were produced from expression constructs encoding C2S1 and VS1 NTD_{258} using *Pst*I restriction sites to exchange fragments containing the region encoding S1 AA 213.

2.2. Recombinant S protein production and purification

Secreted, trimeric strep-tagged recombinant C2S1 and its NTDs and CTDs, C3S1, VS1 and their NTD_{258} proteins, V NTD $_{258}$Y43H, V NTD_{258}S213A as well as C2 S-ectodomain proteins were produced in human embryonic kidney (HEK) 293T cells as described [52, 129, 168]. The tissue culture supernatants were harvested at 6 days post transfection. Protein expression was analyzed by western blot using horseradish peroxidase (HRPO)-conjugated Strep-Tactin (IBA GmbH). The proteins were purified using Strep-Tactin Sepharose columns according to the manufacturer's protocols (IBA GmbH). The concentration of purified proteins was determined by Qubit® 2.0 fluorometer (Invitrogen), then confirmed and normalized by electrophoresis in Mini-PROTEAN®TGX Stain-Free™ Precast Gels (Bio-Rad).

2.3. Protein histochemistry

Sections of formalin-fixed, paraffin-embedded tissues from healthy 60-day old specific-pathogen-free-white leghorn chickens were prepared as described [52]. The binding efficiency to chicken tissue sections was assessed as described [52, 129] with minor modifications: antigen retrieval was conducted at 80° C for 30 min, Tris buffers were substituted for phosphate buffers,

slides were blocked with universal negative serum (Biocare, Pacheco, CA) instead of 10% goat serum, and the addition of most reagents and washing steps were performed by an intelliPATH FLX automated slide stainer (Biocare, Pacheco, CA). S proteins and 3-amino-9-ethyl-carbazole (AEC+; Dako, Carpinteria, CA) were added manually. Briefly, equimolar concentrations of S proteins (100 µg/ml S1, and 54 µg/ml NTDs and CTDs used for comparison with S1, or 50 µg/ml S-ectodomain and 12.5 µg/ml NTDs for comparison with S-ectodomain) pre-complexed with Strep-Tactin-HRP (IBA GmbH, Göttingen, Germany) were incubated with deparaffinized and rehydrated tissue sections overnight at 4 °C. Bound S protein was visualized with AEC+ chromogenic substrate. The tissues were counterstained with hematoxylin and mounted with Lerner AquaMount (Covance, Princeton, NJ). Images were captured from an Olympus BX41 microscope with an Olympus DP71 12 mp camera.

3. Results

3.1. Protein expression

Codon-optimized sequences of the S1 gene of an IBV Ark strain (C2 subpopulation of the ArkDPI IBV vaccine) and its subdomains (NTDs and CTDs) were cloned into the pCD5 expression vector, preceded by sequences encoding the CD5 signal peptide and followed by sequences encoding the GCN4 trimerization domain and Strep-tag II (Fig. 5.1B). Expression constructs encoding the subdomains comprised of AA 19-258 (NTD_{258}), 19-137 (NTD_{137}), 259-538 (CTD_{259}), 258-538 (CTD_{258}) and 138-538 (CTD_{138}), as well as full-length S1 (AA 19-538) and S-ectodomain were transfected into HEK293T cells, and the secreted proteins were confirmed and purified. In addition, NTD_{258} of the C3 vaccine subpopulation, vaccine (V) major population, and V NTD_{258} with amino acid change Y43H or S213A were produced. As seen in

Fig. 5.1C, all S1 subdomain proteins could fold independently into stable proteins and be isolated except CTD_{259} (AA 259-538) (result not shown), which was not detected. Surprisingly, extension of the CTD_{259} by a single amino acid at the amino terminus to generate CTD_{258} (AA 258-538) resulted in expression of a stable secreted protein. Moreover, NTD_{258} of the vaccine subpopulation C3, V, and V with either Y43H or S213A changes were also produced (Fig. 5.1D). Based on their electrophoretic mobility, consistent with previous results of others [42, 52], the recombinant proteins were highly glycosylated.

3.2. RBD of IBV Ark strain is located within the N-terminal 19-258 amino acids of the S1 protein

Protein histochemistry showed that the C2 NTD_{258} protein bound strongly to all chicken tissues tested except lung tissues (Fig. 5.2). In contrast, neither C2 NTD_{137} nor any tested C2 CTDs showed binding to any tissues tested (results not shown). Remarkably, the C2 NTD_{258} protein had higher binding affinity to all chicken tissues tested compared to full-length S1 protein and it even bound to kidney tissue, while the full-length S1 did not bind (Fig. 5.2).

3.3. Binding activity of C2 NTD_{258} compared to C2S1 extended with S2 ectodomain (C2 S-ectodomain)

Because C2 S-ectodomain had previously been shown to also bind chicken tissues better than S1 [165], the binding activity of C2 NTD_{258} protein was compared to S-ectodomain using protein histochemistry. Interestingly, C2 NTD_{258} protein could bind to the epithelium of trachea, nasal mucosa, nasolacrimal gland, conjunctiva, choana and lacrimal duct similarly to S-ectodomain (Fig. 5.3). In contrast, C2 NTD_{258} protein showed somewhat weaker binding affinity to non-respiratory tissues kidney, cecal tonsils, and cloaca compared to S-ectodomain protein

(Fig. 5.3). Additionally, C2 NTD_{258} protein, like S1 protein, was not sufficient to bind to the lung tissue, indicating that S2 is required to allow binding to lung tissues (Fig. 5.3).

3.4. The binding activity of S1 NTDs of ArkDPI vaccine virus subpopulations is associated with their selection

The S1 and NTD_{258} proteins of the ArkDPI vaccine subpopulation C2 (strongly selected in chickens), C3 (weakly selected in chickens), and vaccine major population (negatively selected in chickens) were compared in their binding affinity to different chicken tissues using protein histochemistry. As was found for C2 NTD_{258}, V NTD_{258} showed stronger binding activity to all tissues tested except lung compared to its full-length S1 protein (results not shown). Similarly, C3-NTD_{258} showed binding to all tissues tested except kidney, in contrast to C3 S1, which showed very weak or no binding (results not shown), indicating that NTD residues (19-258) are important for binding. Like C2 CTDs, all tested CTD subdomains of these Ark vaccine subpopulations did not bind to any chicken tissues (results not shown).

Comparison of the binding efficiency of NTD_{258} of two ArkDPI vaccine subpopulations selected in chickens (C2 and C3) as well as the vaccine major population (V NTD_{258}), showed that the S1 NTD_{258} of the strongly selected C2 subpopulation had greater binding to all tested tissues compared to C3 NTD_{258} and V NTD_{258} (Fig 5.4). C3 NTD_{258} bound to the epithelium of nasal mucosa better than V NTD_{258}, while V NTD_{258} bound more to cecal tonsil. C2 and V NTD_{258} were able to bind to kidney tissue, while no binding by C3 NTD_{258} was detected (Fig. 5.4).

3.5. Enhanced binding activity of V NTD_{258} with either Y43H or S213A AA change

To further investigate the effect of each of the two amino differences between V and C2 NTDs on binding, we generated V NTD_{258} Y43H and S213A. Remarkably, V NTD_{258} with either AA change Y43H or S213A showed improved binding to all chicken tissues tested as well as embryonic chorioallantoic membrane (CAM) (Fig. 5.5) compared to V $NTD_{258,}$ and similar binding as C2 $NTD_{258,}$ indicating that the AA change found in C2 at either of these positions is sufficient to allow enhanced binding.

4. Discussion

In this study, we aimed to identify the RBD of Ark serotype IBVs and compare their attachment patterns to their full-length S1 proteins. We chose to first identify the RBD within the S1 protein of the Ark-DPI vaccine C2 subpopulation because the C2 S1 AA sequence is almost identical to that of the unattenuated parent ArkDPI isolate (GenBank accession number AAB61013) [127] and represents the consensus sequence of vaccine subpopulations rapidly positively selected in chickens after vaccination with ArkDPI-derived attenuated vaccines [81-83, 95, 128]. Then we compared the binding affinity of the C2 RBD identified to those of the C3 vaccine subpopulation, found to be weakly selected in chickens, and the negatively-selected vaccine major population. All subdomain proteins of C2, C3 and V-S1 generated were able to fold independently to produce stable proteins that could be isolated except CTD_{259} (AA 259-538), which did not produce a stable secreted protein.

The binding characteristics of these recombinant proteins to different relevant chicken tissues were assessed and compared using protein histochemistry. C2 NTD_{258} protein could bind strongly to all chicken tissues tested except lung tissues, but the shorter NTD_{137} and the

CTDs tested did not bind to any of the tissues. These results are in agreement with those of Promkuntod, et al. [50], who identified the RBD of M41 S protein in the NTD (AA 19-253) and found it was necessary and sufficient for alpha-2,3 sialic acid-dependent binding to chicken respiratory tract. However, although they revealed that the NTD is sufficient to bind in a similar manner as S1, our results showed better binding of Ark NTD_{258} proteins than the full-length S1 proteins in all chicken tissues tested. Moreover, the S1 NTDs of vaccine subpopulation C3 and the vaccine major population showed high binding affinity, whereas their full-length S1 proteins did not bind or bound weakly to most tissues. One explanation for this greater binding of NTDs than S1 is that the S1 CTD might have a role in masking the binding sites in the S1 NTD. Similarly, the HCoV-NL63 S1 CTD, which comprises the RBD, exhibits markedly higher binding activity to its ACE2 receptor compared with that of the full-length S1 domain [169, 170]. HCoV-NL63 S protein structure determined by cryo-electron microscopy [170] suggests that many of the HCoV-NL63 receptor-binding residues in the CTD are buried through interaction with the NTD and thus are not available to engage the host-cell receptor. In contrast, no binding efficiency differences were observed between S1 compared to the RBD of SARS-CoV S [169]. Promunktod, et al. [50], identified specific AA residues within the N-terminal residues 19–69 of M41 S1 that are essential for binding to chicken tissues. However, we found that the short NTD_{137} protein that comprised AA 19-137 did not show binding to any chicken tissues tested, indicating that the residues from 19-137 by themselves are not sufficient for binding and the residues between 138-258 are also required for binding. However, the long CTD_{138} that comprises AA 138-538 did not bind to any tissues. Thus, multiple domains within the NTD might be involved in binding to host tissues.

In addition, we found that C2 NTD_{258} protein bound to all tested tissues better that V NTD_{258} protein, although only two amino acid differences are found within the NTDs, at positions 43 and 213. At these positions, C2 S1 matches the consensus of Ark-type IBV clinical isolates [83]. Interestingly, V NTD_{258} with either single AA change Y43H or S213A allowed enhanced binding of V NTD_{258} to all chicken tissues tested, similar to C2 $NTD_{258,}$ which has both changes. Thus, each of these AA residues is important for enhancing the binding efficiency. Others have demonstrated that the Y43H change within the full-length ArkDPI S1 protein also results in enhanced binding to trachea [159]. Amino acid 43H is one of the four specific amino acid residues identified by Promunktod, et al. [50], to be essential for binding of M41 S1 to chicken trachea. Efficient binding by V NTD_{258} S213A, which has tyrosine at position 43, is in contrast to results of Promunktod, et al., who showed that a single substitution of AA 43 in M41 S1 (H43Q) abolished binding. Furthermore, increased binding of V NTD_{258} Y43H to CAM compared to V NTD_{258} is opposite to the findings of Leyson, et al. [159], who found that the same change in recombinant ArkDPI vaccine S1 abolished detectable binding to CAM.

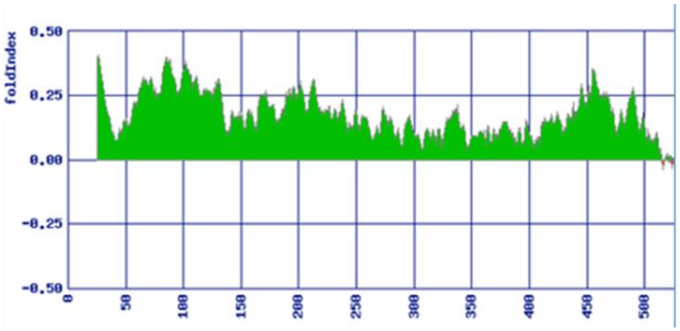
We [165] and others [42] found that the binding efficiency to chicken tissues improved after extension of the S1 subunit with the S2 ectodomain, suggesting the S2 subunit might affect the conformation of the S1 RBD. As the NTD_{258} showed better binding activity than the full-length S1, we compared its binding activity to S-ectodomain protein. Interestingly, NTD_{258} protein bound to most of tissues tested in equivalently to the S-ectodomain. However, NTD_{258}, like S1 protein, was not sufficient to bind to lung tissues, indicating that S2 is required to allow binding to lung tissues and improve the binding to non-respiratory tissues kidney, cecal tonsils and cloaca.

Collectively, our results show that the RBD of IBV ArkDPI-like spike proteins is located in the NTD (AA 19–258) of the S1 protein. The high binding affinity of NTD_{258} protein suggests that its folding structure might be different from that of S1 protein and thus, some efficient binding sites might be hidden within S1. In addition, the AAs 43H and 213A are essential for enhanced binding affinity.

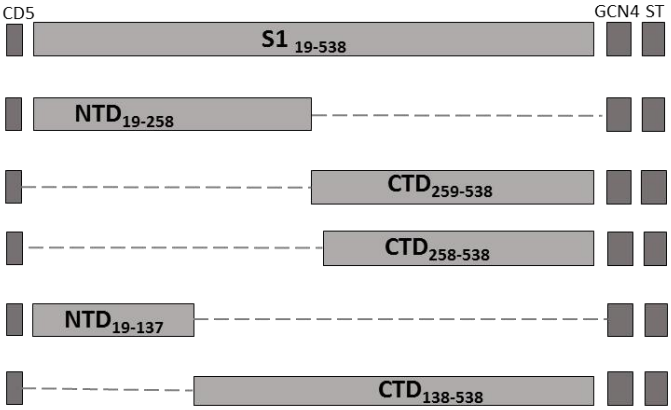
Table 5.1. Primers used for producing S1 NTD and CTD constructs

Primer	Sequence
pCD5S1F-NheI	GCTTCCGTGCTAGCAAACC
C2S1-CdomF	CCGTGCTAGCAACCTTCAGCAACGAGAGC
C2S1Cdom138F	GGCAGCTAGCAATAGAGCACGGCAGCG
C2S1-Cdom258F	CCGTGCTAGCAGTGAAGCACAAGTTCATCGTG
C2S1-Ndom137R	CACAGGTACCCCGGCGGCGATCCGGATG
C2S1NdomR	CACAGGTACCCCGAAGTTGGTCAGGGTCAG
pCD5R	TCCTCGATCTGCTTCATGC

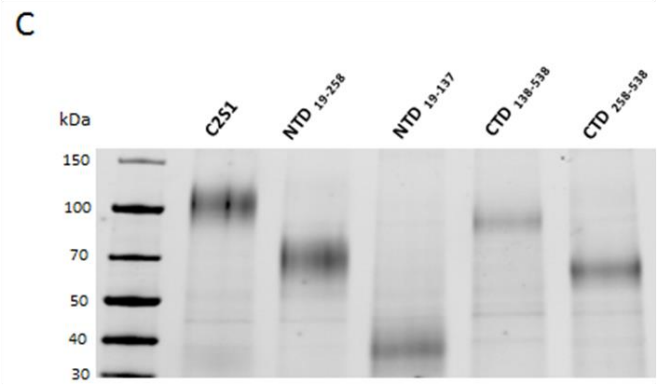
A


foldIndex
0.50
0.25
0.00
-0.25
-0.50
0
50
100
150
200
250
300
350
400
450
500

B


CD5
GCN4 ST
S1 19-538
NTD19-258
CTD259-538
CTD258-538
NTD19-137
CTD138-538

C


kDa
C2S1
NTD 19-258
NTD 19-137
CTD 138-538
CTD 258-538
150
100
70
50
40
30

D

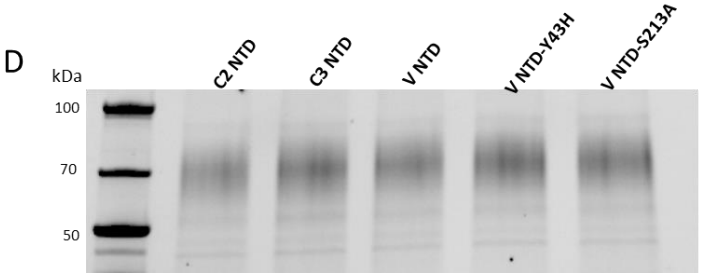
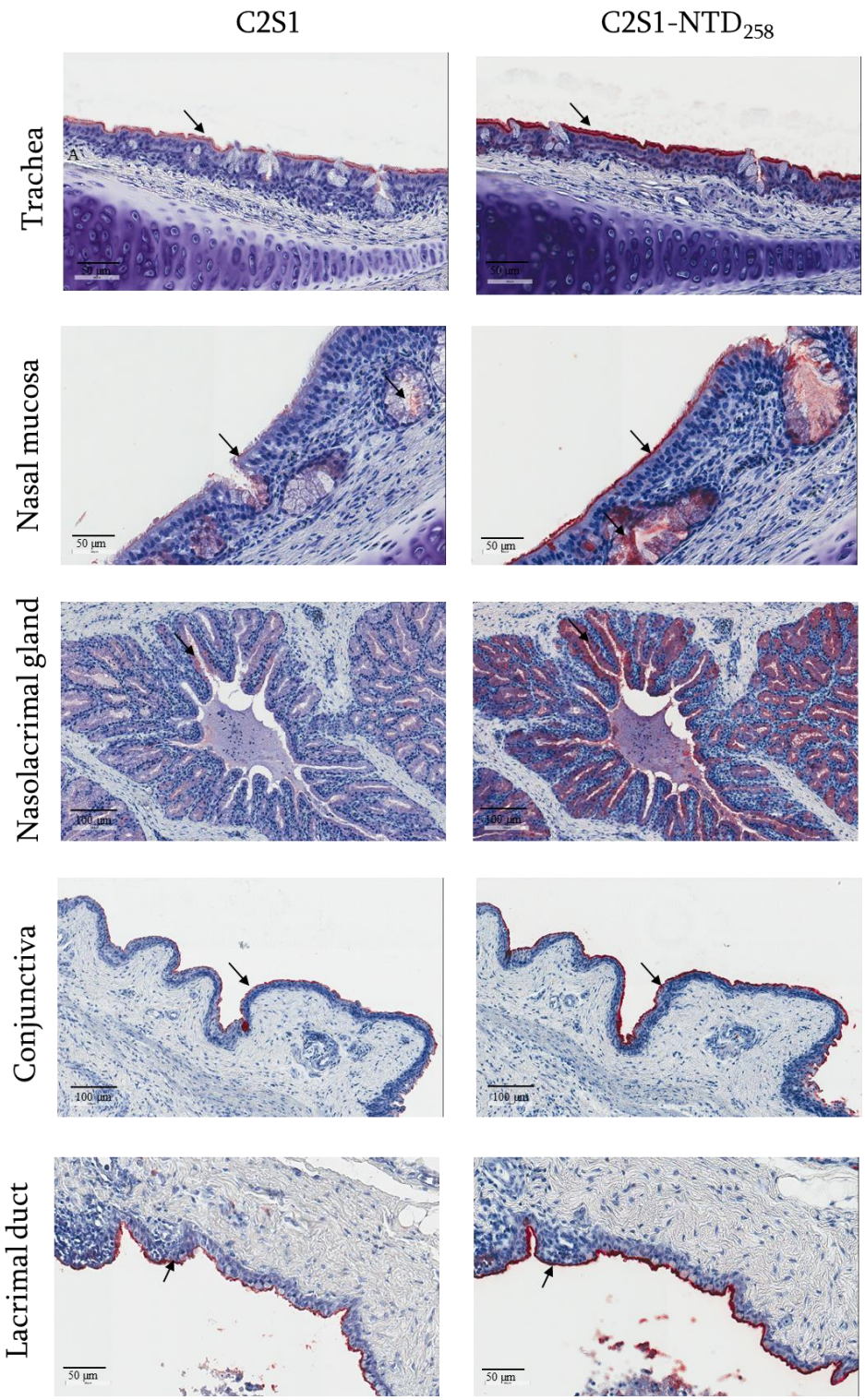

kDa
C2 NTD
C3 NTD
V NTD
V NTD-Y43H
V NTD-S213A
100
70
50

Fig. 5.1. Expression of S1 and S1 subdomains of IBV ArkDPI vaccine subpopulations. (A) Prediction of the folding of S1 protein using FoldIndex (folded region indicated by green and unfolded by red color). Unlike the M41 S1 protein [50], there is no clear predicted unfolded region between the NTD and CTD. (B) Schematic representation of truncated recombinant C2S1 proteins. S1 and subdomains were flanked by an N-terminal CD5 signal sequence and a C-terminal artificial GCN4 trimerization motif and Strep-tag II (ST) for purification and detection of proteins. (C and D) Electrophoresis of purified proteins in Mini-PROTEAN®TGX Stain-Free™ Precast Gels.


C2S1
C2S1-NTD258
Trachea
Nasal mucosa
Nasolacrimal gland
Conjunctiva
Lacrimal duct
50 μm
100 μm

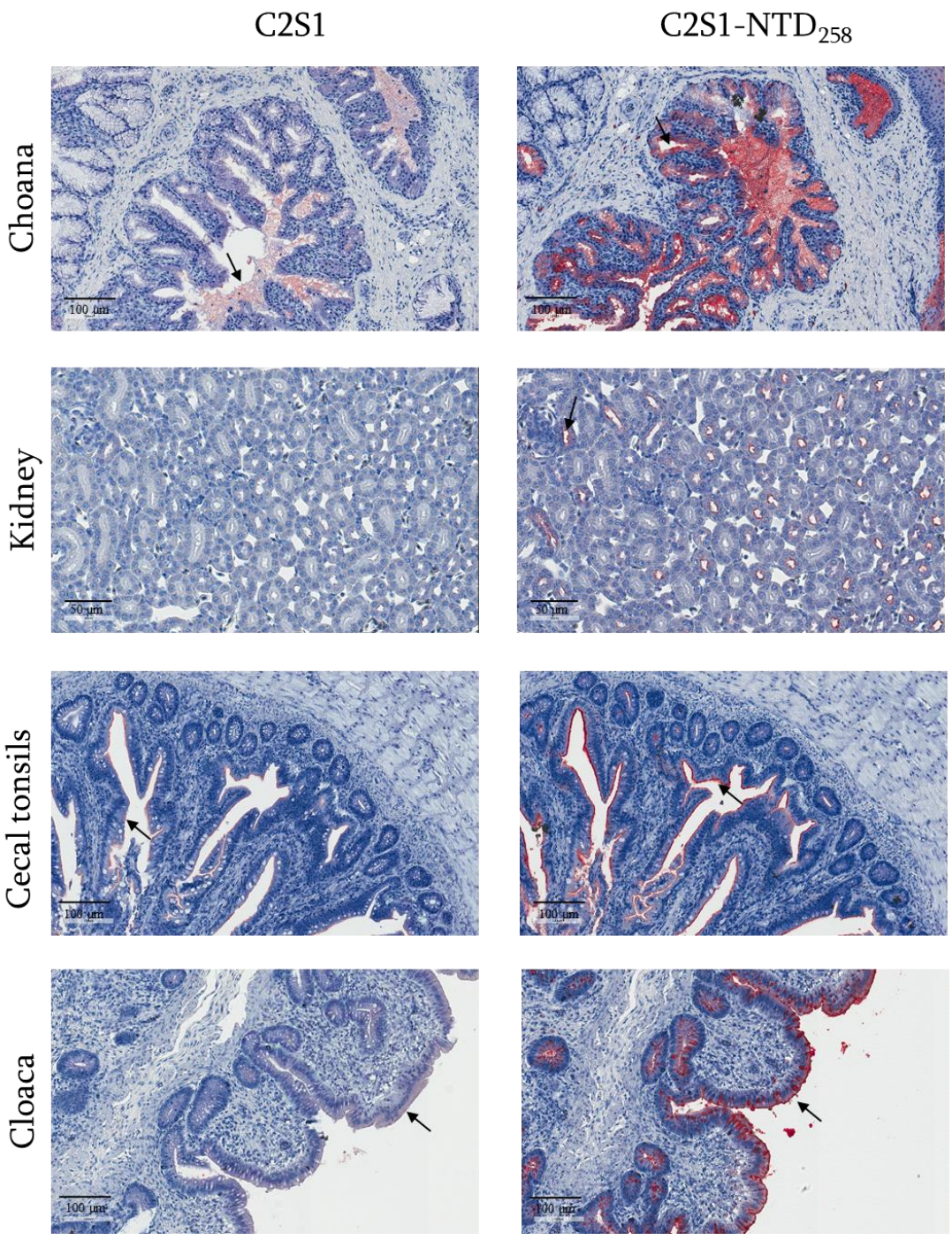
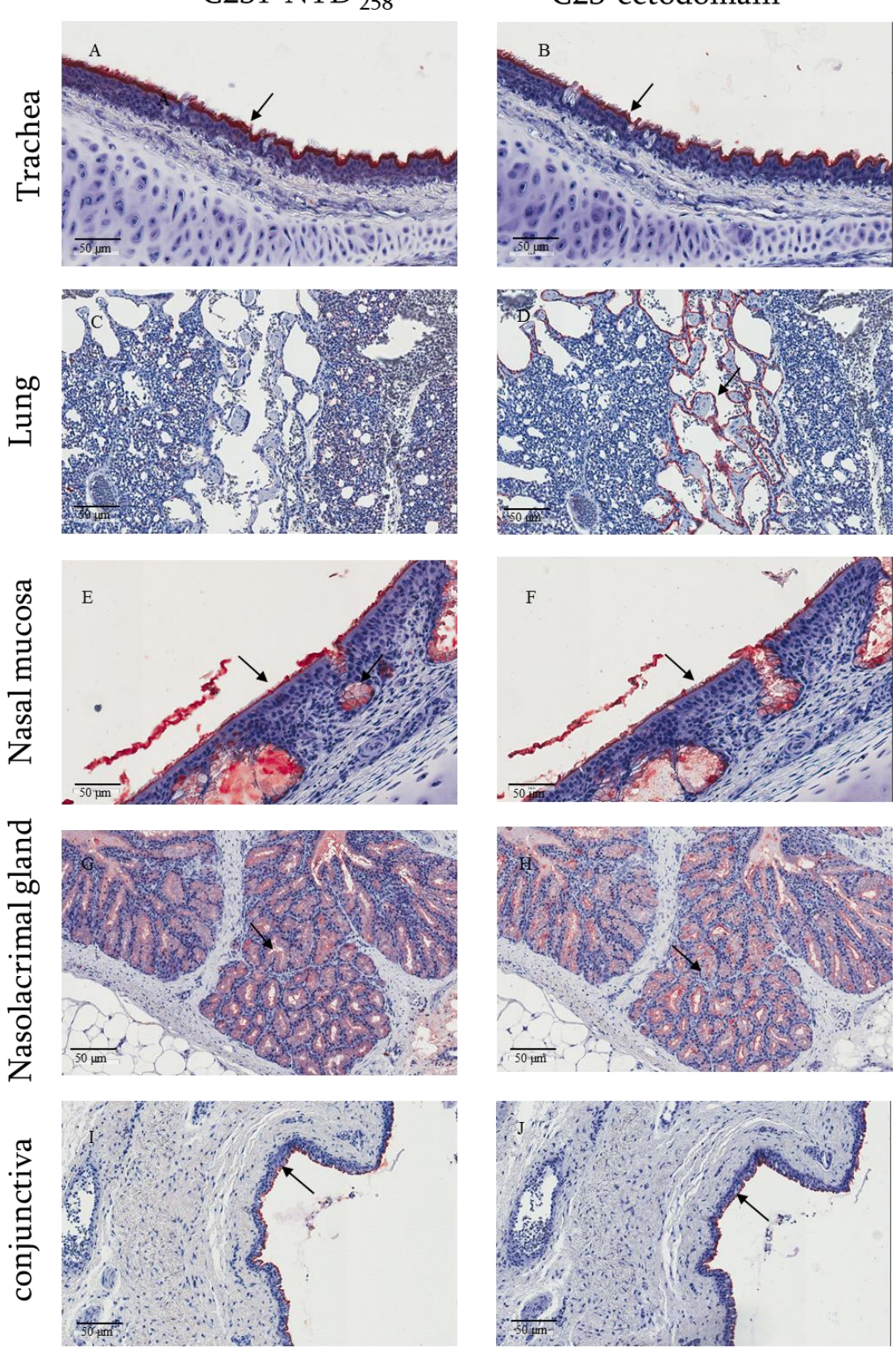


Fig. 5.2. Protein histochemistry comparing the binding of equimolar concentrations ofrecombinant IBV Ark-type C2S1 (A, C, E, G, I, K, M, O & Q) and C2S1-N-terminal

domain AA 19-258 (NTD_{258}) (B, D, F, H, J, L, N, P & R) proteins to chicken tissues. A & B show S1 (A) and NTD_{258} (B) binding to tracheal epithelium, cilia and goblet cells. C & D show the binding to nasal mucosal epithelium and secretory cells. E & F show the binding to nasolacrimal glandular epithelium. G & H show the binding to conjunctiva epithelium and I & J show the binding to the epithelium lining the lacrimal duct. K & L show the binding to glandular epithelium in choana. M & N show that only C2S1-NTD_{258} binds to tubular epithelium of kidney. O & P show the binding to cecal tonsils epithelium and Q & R show binding to cloacal epithelium. Bound spike protein is indicated by red color (arrows).


C2S1-NTD 258
C2S-ectodomain
Trachea
Lung
Nasal mucosa
Nasolacrimal gland
conjunctiva
A
B
C
D
E
F
G
H
I
J
50 µm

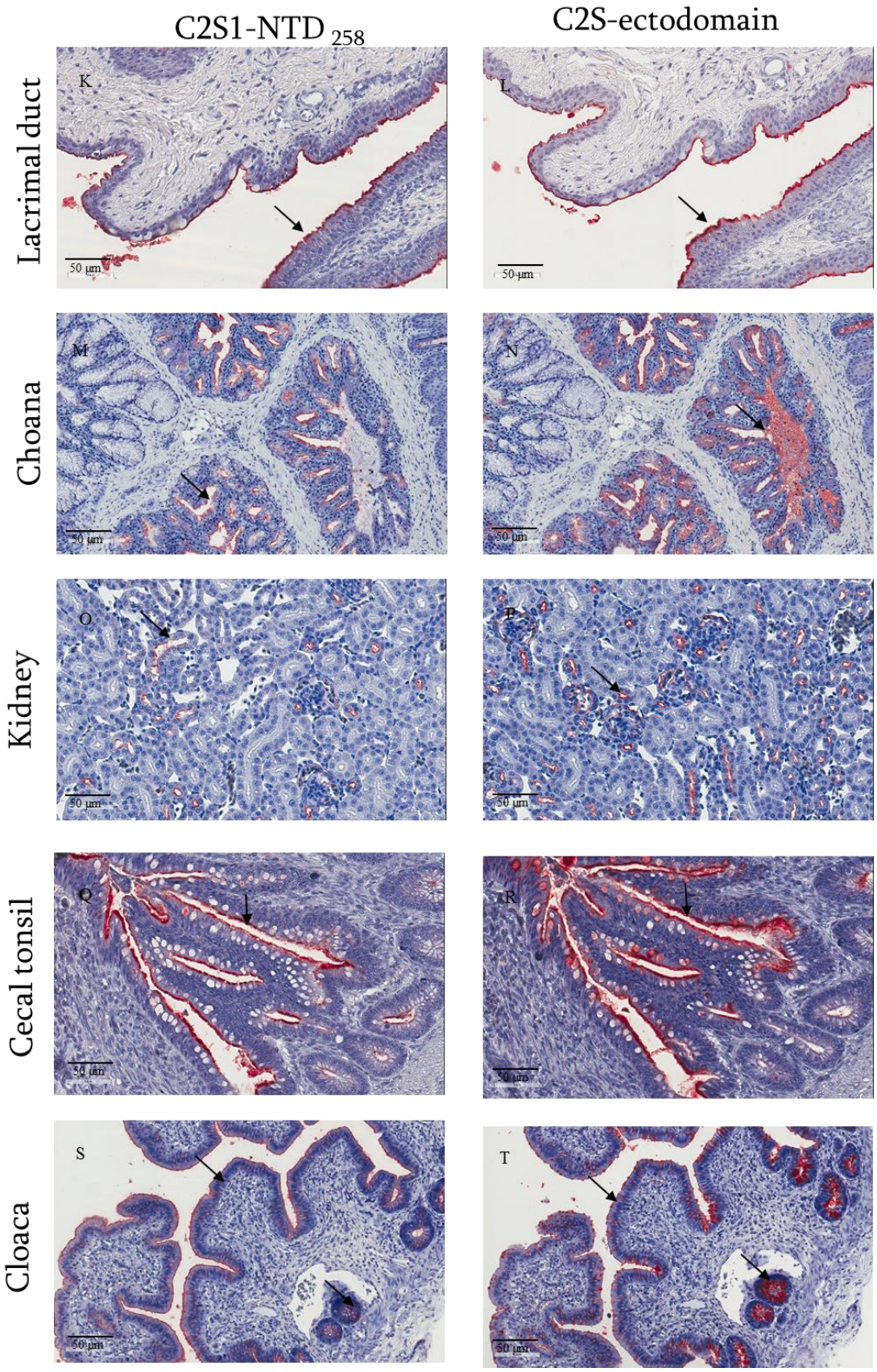
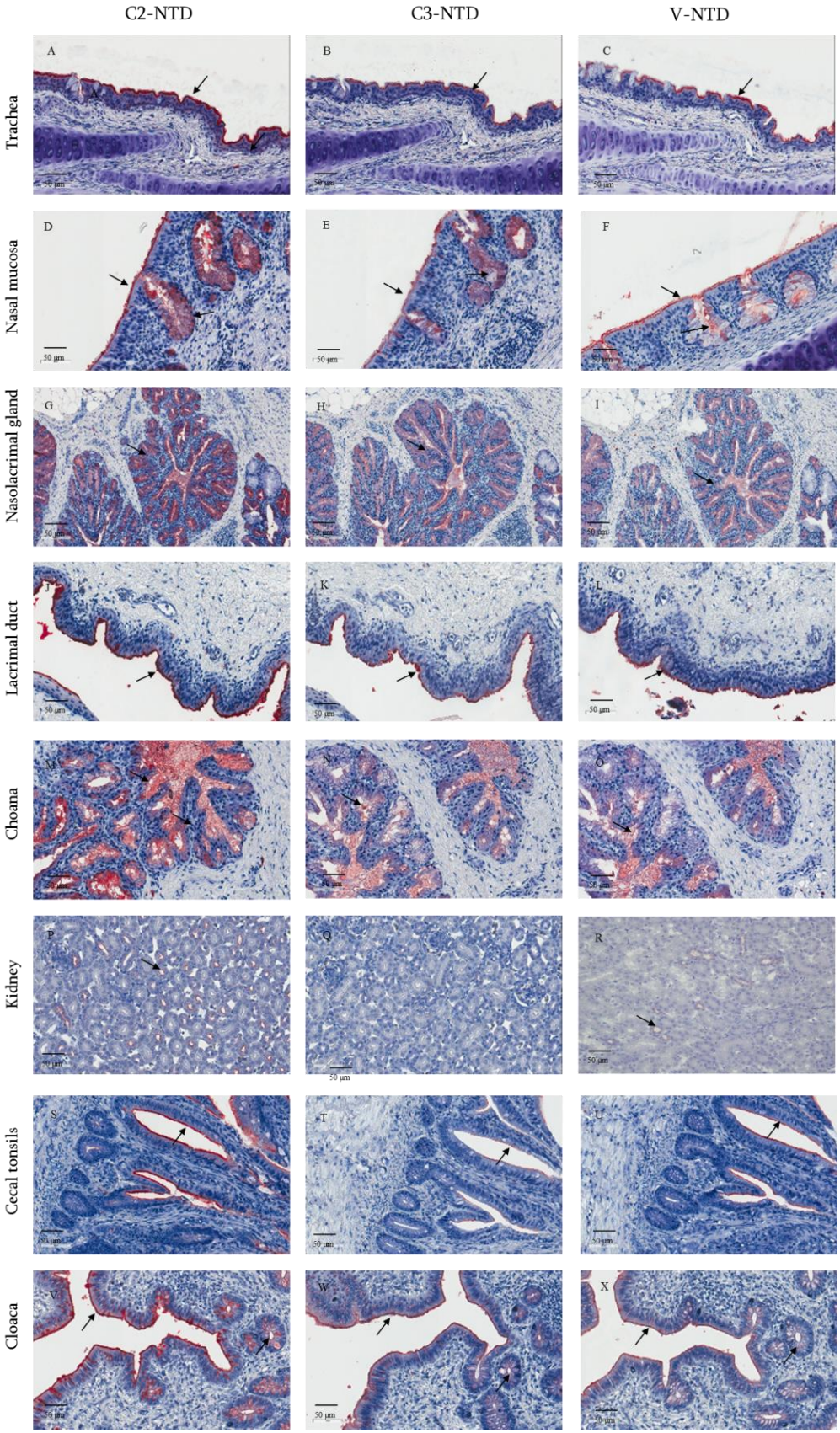

C2S1-NTD 258
C2S-ectodomain
Lacrimal duct
Choana
Kidney
Cecal tonsil
Cloaca
K
L
M
N
O
P
Q
R
S
T
50 µm

Fig. 5.3. Protein histochemistry comparing binding of equimolar concentrations of recombinant IBV Ark-type C2S1-N-terminal domain NTD_{258} (AA 19-258) (A, C, E, G, I, K, M, O, Q, & S) and C2 S-ectodomain (B, D, F, H, J, L, N, P, R, and T) proteins to chicken tissues. A & B show the binding to tracheal epithelium and cilia. C & D show that only S-ectodomain can bind to the alveolar epithelium of lung. E & F show binding to nasal mucosal epithelium and secretory cells. G & H show the binding to nasolacrimal glandular epithelium. I & J show the binding to conjunctiva epithelium and K & L show the binding to the epithelium lining the lacrimal duct. M & N show the binding to glandular epithelium in choana. O & P show that only C2S1-NTD_{258} binds to tubular epithelium of kidney. Q & R show binding to epithelium of cecal tonsils. S & T show binding to cloacal epithelium. Bound spike protein is indicated by red color (arrows).


C2-NTD
C3-NTD
V-NTD
Trachea
Nasal mucosa
Nasolacrimal gland
Lacrimal duct
Choana
Kidney
Cecal tonsils
Cloaca
A
B
C
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N
O
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Q
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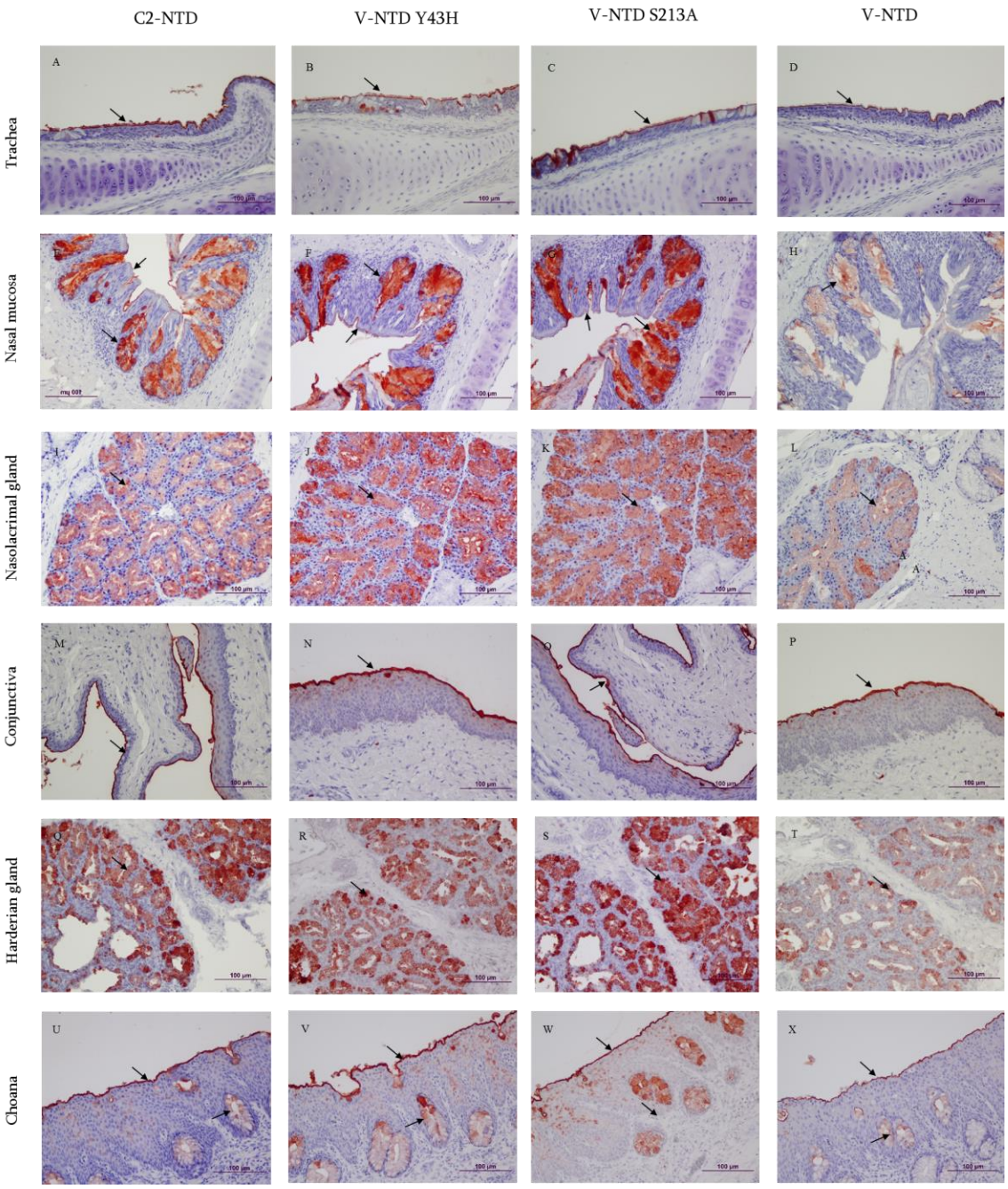
U
V
W
X
50 µm

Fig. 5.4. Protein histochemistry of recombinant S1 NTD_{258} protein of C2 (A, D, G, J, M, P, S, & V) , C3 (B, E, H, K, N, Q, T, & W) ArkDPI-derived vaccine subpopulations, and the major vaccine population (V) (C, F, I, L, O, R, U & X). A, B, & C show the binding to tracheal epithelium and cilia. D, E, & F show the binding to nasal mucosal epithelium and secretory cells. G, H, & I show the binding to nasolacrimal glandular epithelium. J, K, & L show the binding to the epithelium lining the lacrimal duct and M, N, & O show the binding to glandular epithelium in choana. P, Q, & R show that only C2-NTD_{258} and V NTD_{258} bind to tubular epithelium of kidney. S, T, & U show the binding to epithelium of cecal tonsils and V, W, & X binding to cloacal epithelium. Bound spike protein is indicated by red color (arrows).


C2-NTD
V-NTD Y43H
V-NTD S213A
V-NTD
Trachea
Nasal mucosa
Nasolacrimal gland
Conjunctiva
Harderian gland
Choana
A
B
C
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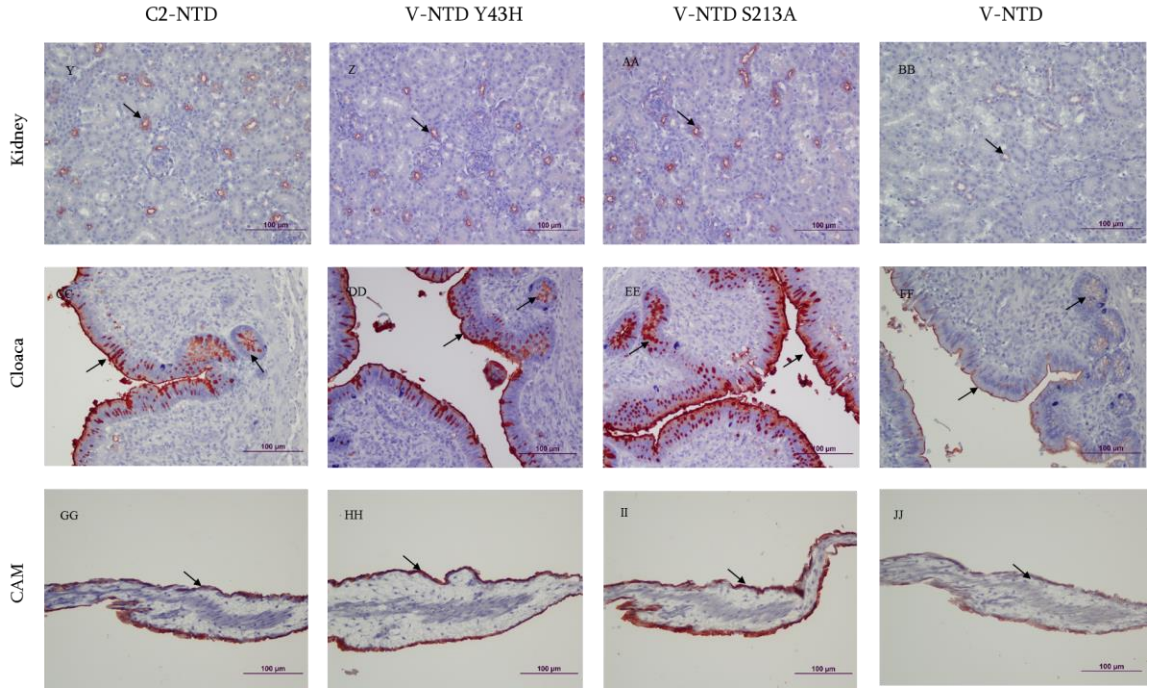

Fig. 5.5. Protein histochemistry of recombinant IBV Ark-type S1 NTD_{258} protein of C2 (A, E, I, M, O, U, Y, CC, GG), vaccine (V) major population with AA changes Y43H (V NTD Y43H) (B, F, J, N, R, V, Z, DD, HH) or S213A (V NTD S213A) (C, G, K, O, S, W, AA, EE, II) and V NTD (D, H, L, P, T, X, BB, FF, JJ). A, B, C, & D show binding to tracheal epithelium. E, F, G, & H show binding to nasal mucosal epithelium and secretory cells. I, J, K, & L show binding to nasolacrimal glandular epithelium. M, N, O, & P show binding to the epithelium of conjunctiva and Q, R, S, & T show binding to Harderian gland and U, V, W, & X show binding to glandular epithelium in choana. Y, Z, AA, & BB show binding to tubular epithelium of kidney. CC, DD, EE, & FF show binding to cloacal epithelium. GG, HH, II, & JJ show binding to embryonic chorioallantoic membrane (CAM). Bound spike protein is indicated by red color (arrows).

CHAPTER 6

Conclusion

In this work, we demonstrated the role of recombinant IBV spike proteins and domains in attachment, vaccine subpopulation selection and protective immunity that reflect on development of new strategies for IBV control.

First, we evaluated the effect of immunization with recombinant Ark S-ectodomain protein compared to S1 protein alone on protection against IBV Ark challenge. Recombinant S-ectodomain protein shows improved binding to respiratory epithelium compared to recombinant S1 protein. Consistent with this observation, recombinant S-ectodomain protein elicits improved protection against IBV Ark-type challenge as determined by antibody responses, viral load, and tracheal lesions. Thus, the S2 domain has an important role in inducing protective immunity and the S-ectodomain provides a promising option for better viral vectored and/or subunit vaccine strategies.

Second, the protective capabilities of the S2 protein of IBV 4/91 expressed from a recombinant Newcastle disease virus LaSota (rLS) were assessed. Although IBV 4/91 (serotype 793/B) vaccine had been shown to protect against divergent IBV strains in a prime-boost approach with IBV Mass vaccine, our demonstration of lack of cross protection by 4/91 S2 protein indicates that this cross protection could not be explained

by the immune response against the conserved S2 protein. Thus, the S2 domain alone might be not enough for inducing broad protection.

Third, the role of spike protein attachment in ArkDPI vaccine subpopulation selection in chickens was examined. The binding efficiency to different chicken tissues of recombinant S1 and S-ectodomain proteins representing C2 (strongly selected) and C3 (weakly selected) ArkDPI-derived vaccine subpopulations was compared with those representing the major vaccine population (negatively selected). Strongly selected C2S1 protein bound to different chicken tissues better than S1 of negatively selected vaccine major population, suggesting a role of S1 in subpopulation selection. C3S1 did not bind to any of tested tissues. None of the S1 proteins bound to lung and kidney tissues. Comparing S1 and S ectodomains, C2 and vaccine major S-ectodomain proteins bound chicken tissues more strongly than the corresponding S1 proteins, and bound to lung and kidney tissues. C3 S-ectodomain bound to all tissues tested, confirming a role for the S2 ectodomain in virus attachment. A role for S2 subunits in subpopulation selection was not demonstrated.

Finally, we identified the receptor-binding domain (RBD) of Ark-type IBV S1 and its contributions to binding efficiency to different relevant chicken tissues. The RBD of IBV Ark strain is located in the NTD (AA 19–258) of S1 protein. The higher binding affinity of S1 NTD protein compared to complete S1 suggests that S1 NTD protein folding structure might be different from S1 protein and thus, some efficient binding sites hidden within S1. Stronger binding of strongly selected C2 S1 NTD to chicken tissues than those of weakly selected C3 and negatively selected vaccine major population

suggests more efficient attachment plays a role in selection. The S1 NTD amino acids 43H or 213A are essential for enhanced binding affinity.

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