Since January 2020 Elsevier has created a COVID-19 resource centre with free information in English and Mandarin on the novel coronavirus COVID-19. The COVID-19 resource centre is hosted on Elsevier Connect, the company's public news and information website.

Elsevier hereby grants permission to make all its COVID-19-related research that is available on the COVID-19 resource centre - including this research content - immediately available in PubMed Central and other publicly funded repositories, such as the WHO COVID database with rights for unrestricted research re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for free by Elsevier for as long as the COVID-19 resource centre remains active.
Protection against infectious bronchitis virus by spike ectodomain subunit vaccine

Fatma Eldemery1, Kellye S. Joiner, Haroldo Toro, Vicky L. van Santen*

Department of Pathobiology, College of Veterinary Medicine, Auburn University, 264 Greene Hall, Auburn, AL 36849-5519, USA

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.

© 2017 Elsevier Ltd. All rights reserved.

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 [2,3], 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 [3,4]. 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 [3,4].

The spike (S) protein of IBV mediates viral entry into host cells [5,6]. Its S1 subunit mediates viral attachment to host cells and induces virus-neutralizing antibodies that are important for host protective immune responses [7–9]. However, the S1 subunit shows extensive amino acid sequence variability among IBV strains, which leads to the virus’s immunological escape [1,10,11]. The S2 subunit of S, responsible for membrane fusion, is more conserved among IBV strains [12]. The N-terminal portion of S2 contains immunodominant regions and a neutralizing
epitope and therefore the S2 protein has been suggested for vaccine development [12,13].

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 [14]. Similarly, three immunizations with KM91 S1 protein expressed by a recombinant baculovirus produced only 50% protection against virulent nephropathogenic KM91 strain challenge [15].

The S1 subunit of IBV is sufficient for attachment [5,16–19] and the S2 portion of coronavirus spike proteins has traditionally been considered to play a role only in subsequent entry [20,21]. However, for a role in 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 [22,23]. 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 [3,4]. Its S1 is almost identical to that of the unattenuated parent ArkDPI isolate [24] and represents the consensus sequence of vaccine subpopulations rapidly positively selected in chickens after vaccination with ArkDPI-derived attenuated vaccines [2–4,25,26]. 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 S-ectodomain (S AA 544–1097) was cloned into the pCD5 vector already containing the S1 domain as described [22]. At the S1/S2 border, the furin cleavage site sequence RRSRR was replaced by GGGVP to avoid cleavage of the full length S-ectodomain [22]. 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 [16].

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 [16,22,27]. 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 [22,27] 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-aminoo-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 105 50% embryo infective doses (EID50) of a virulent IBV Ark-type strain (GenBank accession JN861120) previously characterized [28]. 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 QiAamp 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 [29]. 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 [30]. 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 sample was 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 [31]. Briefly, ELISA plates (Nunc MaxiSorp Immuno Plates; Thermo Scientific) were coated with heat-inactivated IBV (ArkDPI vaccine strain; S AA sequence GenBank #ABY6634) purified as described [31]. Individual chicken sera diluted 1:100 were loaded and plates incubated at 4 °C overnight. IBV-specific IgG was detected using biotinylated monoclonal antibody (clone G-1) from 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. 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). 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. 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. 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. 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 S1 protein.
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 [22]. Furthermore, the extension of the M41 S1 with the M41 S2 ectodomain domain increased binding to chicken trachea [23]. 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 [22]. 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. [32,33], 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 [8,34–36]. 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 [14] 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 [13] 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 [37]. The adjuvant used has been reported to stimulate both antibody and cell-mediated immune responses [38–40]. 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 [41].

Fig. 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. 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 25th and 75th 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).
tion, and tissue binding protocols. Fatma Eldemery was sponsored by a scholarship from the Egyptian government (Egyptian Cultural and Educational Bureau, Washington, DC). Research was funded by United States Department of Agriculture’s PRD-CAP grant 2014-08054 and State of Alabama Animal Health and Disease Research.

Conflict of interest

The authors have no conflict of interest to declare.

References

[1] Toro H, Jackwood MW, van Santen VL. Genetic diversity and selection regulates evolution of infectious bronchitis virus. Avian Dis 2012;56:449–55.
[2] Toro H, Pennington D, Gallardo RA, van Santen VL, van Ginkel FW, Zhang J, et al. Infectious bronchitis virus subpopulations in vaccinated chickens after challenge. Avian Dis 2012;56:501–8.
[3] van Santen VL, Toro H. Rapid selection in chickens of subpopulations within AVIBV-derived infectious bronchitis virus vaccines. Avian Pathol 2008;37:293–306.
[4] Gallardo RA, van Santen VL, Toro H. Host intraspatial selection of infectious bronchitis virus populations. Avian Dis 2010;54:807–13.
[5] Cavanagh D. Coronavirus avian infectious bronchitis virus. Vet Res 2007;38:281–97.
[6] Belouzard S, Millet JK, Licitra BN, Whittaker GR. Mechanisms of coronavirus cell entry mediated by the viral spike protein. Viruses 2012;4:1011–33.
[7] Cavanagh D, Davis PJ, Darbyshire JH, Peters RW. Coronavirus IBV: virus retaing spike glycoprotein S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. J Gen Virol 1986;67:1435–42.
[8] Cavanagh D, Davis PJ, Moekett AP. Amino acids within hypervariable region 1 of avian coronavirus IBV (Massachusetts serotype) spike glycoprotein are associated with neutralization epitopes. Virus Res 1988;11:141–50.
[9] Moore KM, Jackwood MW, Hiil DA. Identification of amino acids involved in a serotype and neutralization specific epitope within the S1 subunit of avian infectious bronchitis virus. Arch Virol 1997;142:2249–56.
[10] Kusters JC, Niesters HG, Bleumink-Pluym NM, Davelaar FG, Horzinek MC, Van der Zeijst BA. Molecular epidemiology of infectious bronchitis virus in the Netherlands. J Gen Virol 1987;68:343–52.
[11] Kusters JC, Niesters HG, Lenstra JA, Horzinek MC, van der Zeijst BA. Phylogeny of antigenic variants of avian coronavirus IBV. Virology 1989;169:217–21.
[12] Toro H, Zhao W, Breedlove C, Zhang Z, Yu Q, van Santen V. Infectious bronchitis virus S2 expressed from recombinant virus confers broad protection against challenge. Avian Dis 2014;58:83–9.
[13] Lenstra JA, Kusters JC, Koch G, van der Zeijst BA. Antigenicity of the peplomer protein of infectious bronchitis virus. Mol Immunol 1989;26:7–15.
[14] Ignjatovic J, Gali L. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. Arch Virol 1994;138:117–34.
[15] Song CW, Lee YJ, Lee CW, Song HW, Kim JH, Mo JP, et al. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. J Gen Virol 1998;79:719–23.
[16] Wickramasinghe PN, de Vries RP, Crone A, de Haan CA, Verheije MH. Binding of avian coronavirus spike proteins to host factors reflects virus tropism and pathogenicity. J Virol 2011;85:8903–12.
[17] Promkuntod N, van Eijndhoven RE, de Vrieze G, Crone A, Verheije MH. Mapping of the receptor-binding domain and amino acids critical for attachment in the spike protein of avian coronavirus infectious bronchitis virus. Virology 2014;448:26–32.
[18] Mork AK, Hesse M, Abd El Rahman S, Rautenschlein S, Herrler G, Winter C. Differences in the tissue tropism to chicken oviduct epithelial cells between avian coronavirus IBV strains QX and B1648 are not related to the sialic acid binding properties of their spike proteins. Vet Res 2014;45:67.
[19] Shahwan K, Hesse M, Mork AK, Herrler G, Winter C. Sialic acid binding properties and proteolytic susceptibility of the spike proteins of infectious bronchitis virus strains isolated from different geographical origins. Virus Res 2013;15:1924–33.
[20] Bosch BJ, van der Zee R, de Haan CA, Rattier PJ. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. J Virol 2003;77:8801–11.
[21] Heald-Sargent T, Gallagher T. Ready, set, fuse! The coronavirus spike protein and acquisition of fusion competence. Viruses 2012;4:557–80.
[22] Promkuntod N, Wickramasinghe IN, de Vrieze G, Crone A, Verheije MH. Contributions of the S2 spike ectodomain to attachment and host range of infectious bronchitis virus. Virus Res 2013;177:127–37.
[23] Wickramasinghe IN, van Beurden SJ, Weerts EA, Verheije MH. The avian coronavirus spike protein. Virus Res 2014;194:37–48.
[24] Keefer C, Reed KL, Nix W, Gelb J. Serotype identification of avian infectious bronchitis virus by RT-PCR of the peplomer (S1) gene. Avian Dis 1998;42:275–84.

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.

Acknowledgments

We thank Cassandra Breedlove Kitchens, Natalie Petrenko, Steven Gulley and Cynthia Hutchinson for excellent technical assistance, and Dr. M. Hélène Verheije (Utrecht University) and members of her lab for constructing the S1 expression vector and teaching us their recombinant spike protein production, purification, and tissue binding protocols. Fatma Eldemery was sponsored by a scholarship from the Egyptian government (Egyptian Cultural and Educational Bureau, Washington, DC). Research was funded by United States Department of Agriculture’s PRD-CAP grant 2014-08054 and State of Alabama Animal Health and Disease Research.

Conflict of interest

The authors have no conflict of interest to declare.
[25] McKinley ET, Hilt DA, Jackwood MW. Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. Vaccine 2008;26:1274–84.

[26] Ndegwa EN, Joiner KS, Toro H, van Ginkel FW, van Santen VL. The proportion of specific viral subpopulations in attenuated Arkansas Delmarva poultry industry infectious bronchitis vaccines influences vaccination outcome. Avian Dis 2012;56:642–53.

[27] Wickramasinghe IN, Verheije MH. Protein histochemistry using coronavirus spike proteins: studying binding profiles and sialic acid requirements for attachment to tissues. Methods Mol Biol 2015;1282:155–63.

[28] Gallardo RA, Hoerr FJ, Berry WD, van Santen VL, Toro H. Infectious bronchitis virus in testicles and venereal transmission. Avian Dis 2011;55:255–8.

[29] Callison SA, Hilt DA, Boynton TO, Sample BF, Robison R, Swayne DE, et al. Development and evaluation of a real-time taqman RT-PCR assay for the detection of infectious bronchitis virus from infected chickens. J Virol Methods 2006;138:60–5.

[30] Toro H, van Santen VL, Li L, Lockaby SB, van Santen E, Hoerr FJ. Epidemiological and experimental evidence for immunodeficiency affecting avian infectious bronchitis. Avian Pathol 2006;35:455–64.

[31] Orr-Burks N, Gulley SL, Toro H, van Ginkel FW. Immunoglobulin A as an early humoral responder after mucosal avian coronavirus vaccination. Avian Dis 2014;58:279–86.

[32] Pre-fusion structure of a human coronavirus spike protein. Nature 2016;531:118–21.

[33] Walls AC, Tortorici MA, Bosch B, Frenz B, Rottier PJ, DiMaio F, et al. Cryo-electron microscopy structure of a coronavirus spike glycoprotein trimer. Nature 2016;531:114–7.

[34] Kant A, Koch G, van Roozelaar DJ, Kusters JC, Poelwijk FA, van der Zeijst BA. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. J Gen Virol 1992;73:591–6.

[35] Karaca K, Naqi S, Gelb J. Production and characterization of monoclonal antibodies to three infectious bronchitis virus serotypes. Avian Dis 1992;36:903–15.

[36] Kusters JC, Sturk JA, Koch G, Posthumus WP, Meloen RH, et al. Analysis of an immunodominant region of infectious bronchitis virus. J Immunol 1989;143:2692–8.

[37] Ignjatovic J, Sapats S. Identification of previously unknown antigenic epitopes on the S and N proteins of avian infectious bronchitis virus. Arch Virol 2005;150:1813–31.

[38] Jang SI, Kim DK, Lillehoj HS, Lee SH, Lee KW, Bertrand F, et al. Evaluation of Montanide ISA 71 VG adjuvant during profilin vaccination against experimental coccidiosis. PLoS One 2013;8:e59786.

[39] Jang SI, Lillehoj HS, Lee SH, Lee KW, Lillehoj EP, Bertrand F, et al. Montanide ISA 71 VG adjuvant enhances antibody and cell-mediated immune responses to profilin subunit antigen vaccination and promotes protection against Eimeria acervulina and Eimeria tenella. Exp Parasitol 2011;127:178–83.

[40] Ben Arous A, Devillea S, Paie J, Baksib S, Bertrand F, Dupuisa L. Reduction of Newcastle disease vaccine dose using a novel adjuvant for cellular immune response in poultry. Procedia Vaccinol 2013;7:28–33.

[41] Yin L, Zeng Y, Wang W, Wei Y, Yue C, Cao Y. Immunogenicity and protective efficacy of recombinant fusion proteins containing spike protein of infectious bronchitis virus and hemagglutinin of H3N2 influenza virus in chickens. Virus Res 2016;223:206–12.