



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.



# Immunogenicity and protective efficacy of recombinant fusion proteins containing spike protein of infectious bronchitis virus and hemagglutinin of H3N2 influenza virus in chickens



Lijuan Yin<sup>a</sup>, Yuyao Zeng<sup>a</sup>, Wei Wang<sup>b</sup>, Ying Wei<sup>a</sup>, Chunyi Xue<sup>a</sup>, Yongchang Cao<sup>a,\*</sup>

<sup>a</sup> State Key Laboratory of Biocontrol, School of Life Sciences, Sun Yat-sen University, Guangzhou, 510006, PR China

<sup>b</sup> Linyi Institute for Food and Drug Control, Linyi, Shandong Province, PR China

## ARTICLE INFO

### Article history:

Received 30 April 2016

Received in revised form 9 July 2016

Accepted 29 July 2016

Available online 3 August 2016

### Keywords:

IBV

S1 protein

H3N2

Fusion proteins

Immunogenicity

## ABSTRACT

Infectious bronchitis (IB) is an acute and highly contagious viral respiratory disease of chickens and vaccination is the main method for disease control. The S1 protein, which contains several virus neutralization epitopes, is considered to be a target site of vaccine development. However, although protective immune responses could be induced by recombinant S1 protein, the protection rate in chickens was still low (<50%). Here, we generated fused S1 proteins with HA2 protein (rS1-HA2) or transmembrane domain and cytoplasmic tail (rS1-H3(TM)) from hemagglutinin of H3N2 influenza virus. After immunization, animals vaccinated with fusion proteins rS1-HA2 and rS1-H3(TM) demonstrated stronger robust humoral and cellular immune responses than that of rS1 and inactivated M41 vaccine. The protection rates of groups immunized with rS1-HA2 (87%) were significantly higher than the groups inoculated with rS1 (47%) and inactivated M41 vaccine (53%). And chickens injected with rS1-H3(TM) had similar level of protection (73%) comparing to chickens vaccinated with rS1 (47%) ( $P=0.07$ ). Our data suggest that S1 protein fused to the HA2 or TM proteins from hemagglutinin of H3N2 influenza virus may provide a new strategy for high efficacy recombinant vaccine development against IBV.

© 2016 Elsevier B.V. All rights reserved.

## 1. Introduction

Avian infectious bronchitis (IB) was first described in 1931 as a highly contagious disease and thereafter was found to be caused by an infectious bronchitis virus (IBV), which belongs to *Coronavirus* genus within the *Coronaviridae* (Cavanagh, 2007; Sjaak et al., 2011). Though both live-attenuated and inactivated vaccines are used worldwide to control the disease, IBV occasionally outbreaks in endemic areas (Bijlenga et al., 2004; Cavanagh, 2003). The use of attenuated live-vaccines elicits local, systemic and cell-mediated immunity to the virus (Cavanagh, 2003; Raj and Jones, 1997), but poses a risk of residual pathogenicity associated with vaccine back-passage in flocks (Abro et al., 2012; McKinley et al., 2008; McKinley et al., 2011). Though the inactivated IB vaccines are relatively safe, they are efficacious only when used as boosters after priming vaccination with live vaccines (Ladman et al., 2002). Thus to develop a

safer, more efficacious and economic vaccine candidate is of great interest for scientific research.

Infectious bronchitis virus (IBV) is an enveloped virus containing a single-stranded positive-sense RNA genome of 27.6 kb (Wickramasinghe et al., 2014). The genome of IBV encodes four structural proteins, including spike (S), membrane (M), envelope (E), and nucleocapsid (N) proteins (Cavanagh, 2007). People have found that the spike (S) protein, the largest structural protein, constitutes the characteristic club-shaped 16–21 nm projections that emerge from the virion surface and presents a corona-like appearance under electron microscopy (Cook et al., 2012). In addition, the S protein was suggested to have haemagglutinating activity that the entry of the viruses is mediated by sialic acid binding activity of the S protein (Schultze et al., 1992).

The S protein is a class I fusion peptide, in which the variable S1 domain is involved in host cell attachment and the conserved S2 domain mediates fusion of the virion and cellular membranes (Bosch et al., 2003). The S1 protein contains the primary neutralizing epitopes that can induce neutralization, hemagglutination inhibition (HI) and serotype-specific antibodies (Cavanagh et al., 1986a,b; Ignjatovic and Galli, 1994; Ignjatovic and Galli, 1995; Kant

\* Corresponding author at: School of Life Sciences, Sun Yat-sen University, Higher Education Mega Center, Guangzhou, 510006, China.  
E-mail address: [caoych@mail.sysu.edu.cn](mailto:caoych@mail.sysu.edu.cn) (Y. Cao).

et al., 1992; Koch et al., 1990), thus making it a main target when designing new IBV vaccines.

The S protein alone is considered to be sufficient to induce good immunity (Cavanagh, 2007). However, when the S1 subunit of IBV was recombined using baculovirus, although protective immune responses were induced with multiple inoculations, the percentage of protected chickens was still less than 50% (Song et al., 1998). Thus, to induce better immunogenicity against IBV infections, manipulation of the S protein is considered to be a good strategy when designing new IBV vaccines.

Our previous research demonstrated that various influenza H1, H5 and H9 hemagglutinins (HAs) proteins containing replaced H3-WT TM showed increased thermal stability and immunogenicity (Liu et al., 2014). It is intriguing for us to know whether recombinant hemagglutinin and S protein can also induce better immunogenicity and protective efficacy against IBV infections. In this study, we first investigated whether the recombinant S protein could induce better immune responses. Therefore, we generated a recombinant S1 (rS1) protein and two recombinant S1 fusion proteins, rS1-H3(TM) (fused with the transmembrane (TM) and cytoplasmic tail (CT) of influenza H3N2 HA protein) and rS1-HA2 (fused with the HA2 domain of influenza H3N2 HA protein). Our results suggest that the immunogenicity and protection efficacy of these recombinant fusion proteins have been enhanced in chickens.

## 2. Materials and methods

### 2.1. Cell line and viruses

Sf9 insect cells were cultured in serum-free SF900II medium (GIBCO, Grand Island, NY, USA) at 27 °C. The virulent M41 strain (China Institute of Veterinary Drug Control, IVDC) was propagated in 10-day-old specific pathogen free (SPF) chicken embryos. EID<sub>50</sub> for the IBV M41 was calculated according to the Reed-Muench method as described previously (Reed and Muench, 1938). The A/swine/Guangdong/01/1998(H3N2) was isolated and maintained by our laboratory.

### 2.2. Generation of expression constructs, recombinant baculovirus generation and infection

All primers used in this study were synthesized by Invitrogen and summarized in Table 1. A Bac-to-Bac baculovirus expression system was used for the production of recombinant expression bacmids and baculoviruses. Briefly, IBV-M41 S1 gene, H3(TM) and HA2 fragments (GenBank accession number FJ830855.1) from H3N2 strain (A/swine/Guangdong/01/1998) were first amplified and cloned into pMD-18T vector (Takara) and then amplified sequentially. S1-H3(TM) and S1-HA2 fusion genes were then generated by overlapped PCR and cloned between the Sall and HindIII sites of pFastBac1 vector (Invitrogen) to generate recombinant shuttle plasmids (Fig. 1A). The shuttle vectors were then chemically transformed into competent DH10Bac<sup>TM</sup> Escherichia coli cells (Invitrogen). All clones were verified by sequencing (Invitrogen). Recombinant baculovirus generation and infection were performed as previously described (Liu et al., 2013). Briefly, the obtained recombinant bacmids were transfected into Sf9 cells and incubated for 3 days. The target recombinant baculoviruses (rBVs) were then harvested from the supernatant. The structure of the recombinant fusion proteins are shown in Fig. 1B.

### 2.3. Western blotting analysis

Cell lysates were separated on 10% SDS polyacrylamide gels, and then electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked

and subsequently detected with chicken polyclonal sera (China Institute of Veterinary Drugs Control) against IBV virus at a 1:2000 dilution, horseradish peroxidase (HRP)-conjugated anti-chicken secondary antibody at a 1:5000 dilution (PTGLAB, USA) and commercial ECL kit (Pierce).

### 2.4. Preparation of recombinant proteins and whole inactivated M41 virus for vaccines

For recombinant proteins expression, Sf9 cells were infected with recombinant or wild-type baculoviruses and cultured for 3 days, and the cells were collected, ultrasonicated, and then centrifuged at 12,000 × g for 30 min at 4 °C. The supernatants were further centrifuged at 120,000 × g for 3 h at 4 °C. The resulting precipitates were resuspended in PBS and loaded onto a discontinuous sucrose gradient of 30%, 40%, 50%, and 60% sucrose for recombinant proteins enrichment, and ultracentrifuged at 65,000 × g for 16 h at 4 °C. Fractions were collected from the gradient interphases as described previously (Liu et al., 2014), and their recombinant proteins contents were analyzed using Coomassie Blue stained SDS-PAGE electrophoretogram. Formalin-inactivated M41 virus was purified and concentrated by ultracentrifugation as previously described (Kong et al., 2010).

### 2.5. Determination of protein concentration

The overall protein concentrations of recombinant proteins and inactivated IBV were determined using BCA protein assay kit (Pierce, Company); and the protein concentrations of the three recombinant proteins (rS1 protein, rS1-H3(TM) protein and rS1-HA2 protein) and S1 protein concentration of inactivated IBV were determined using SDS-PAGE gel electrophoresis by GeneSnap and GeneTools from SynGene software with BSA as standard.

### 2.6. Animals, immunization, and viral challenge

Ninety 10-day-old SPF chickens were collected from the SPF Experimental Animal Center (Dahuanong Animal Health Products Co., Ltd., Guangdong, China), and housed in individual isolators under positive pressure, then randomly divided into six groups (n = 15 chickens/group). Recombinant proteins, inactivated M41 virus, Sf9 cell lysate (infected by wild-type baculoviruses), and PBS were emulsified with Montanide<sup>TM</sup> ISA 71 VG at a 3:7 ratio respectively (w:w; antigens:adjuvant) as recommended by the manufacturer (Seppic, Paris, France) and then were used in intramuscular injection. Group 1 chickens were immunized with 5 μg rS1 protein per chicken. Group 2 chickens were injected with 5 μg rS1-H3(TM) protein per chicken. Group 3 chickens were vaccinated with 5 μg rS1-HA2 protein per chicken. Group 4 chickens received 5 μg inactivated M41 (S1 protein) per chicken, as a positive control. For negative controls, animals in the remaining two groups were injected with either rWT (Sf9 cell lysate infected by wild-type baculoviruses) or PBS. Booster immunization, using the same antigen at inoculation, was conducted 2 weeks after the prime.

Two weeks after booster immunization, chickens from each group were challenged with  $2 \times 10^4$  EID<sub>50</sub> of the IBV-M41 strain via the nasal-ocular route in 200 μl PBS per chicken, and were observed daily for clinical symptoms over a week period and euthanized at 7 day after challenge. The trachea and kidney tissues of chickens in all groups were harvested for further detection of virus. All animal experiments were conducted in compliance with the institutional guidelines for animal protection rights in China.

**Table 1**  
Details of PCR primers used in cloning.

Primer pairs	Sequence(5' to 3')	Fragment size(bp)
S1	F: CCGCTCGACATGTTGGTAACACCTCTTTACTA R: <u>GCCAAGCTTT</u> CAACGCTAAAACGACGTGTTC	1614
S1-1	F: CCGCTCGACATGTTGGTAACACCTCTTTACTA R: TCAGTTCAACACGCTCTAAAACGACGTGTTCAT	1611
H3(TM)	F: TTTTAGACGTGTTGAACTGAAGTCAGGATACAA R: <u>GCCAAGCTTT</u> CAGATGCAAATGTGCACCTAAT	141
S1-2	F: CCGCTCGACATGTTGGTAACACCTCTTTACTAG R: CGAATATGCCACGCTCTAAAACGACGTGTTCAT	1611
HA2	F: TTTTAGACGTGGCATATTCGGCGCAATAGCAGG R: <u>GCCAAGCTTT</u> CAGATGCAAATGTGCACCTAAT	666

Restriction enzyme cutting sites (Sall and HindIII) are underlined. F means forward primer, R means reverse primer.

### 2.7. Enzyme-linked immunosorbent assay (ELISA)

Sera were collected from chickens at 0, 14 and 28 days after primary immunization for IBV-specific antibody detection. The purified M41 virosomes were made following the procedures as previously described (Liu et al., 2013), and used as antigen to detect the IBV-specific antibodies in an indirect ELISA. Briefly, the purified virosomes at a concentration of 3.5ug/ml were coated, and incubated with serial dilutions of each serum sample (37 °C for 1 h). The secondary donkey anti-chicken HRP-conjugated antibodies (PTGLAB, USA) were used at a 1:5000 dilution. The optical density value was 450 nm. The end-point titer was determined as the reciprocal of the final dilution giving a threefold optical absorbance of negative control as described previously (Liu et al., 2014).

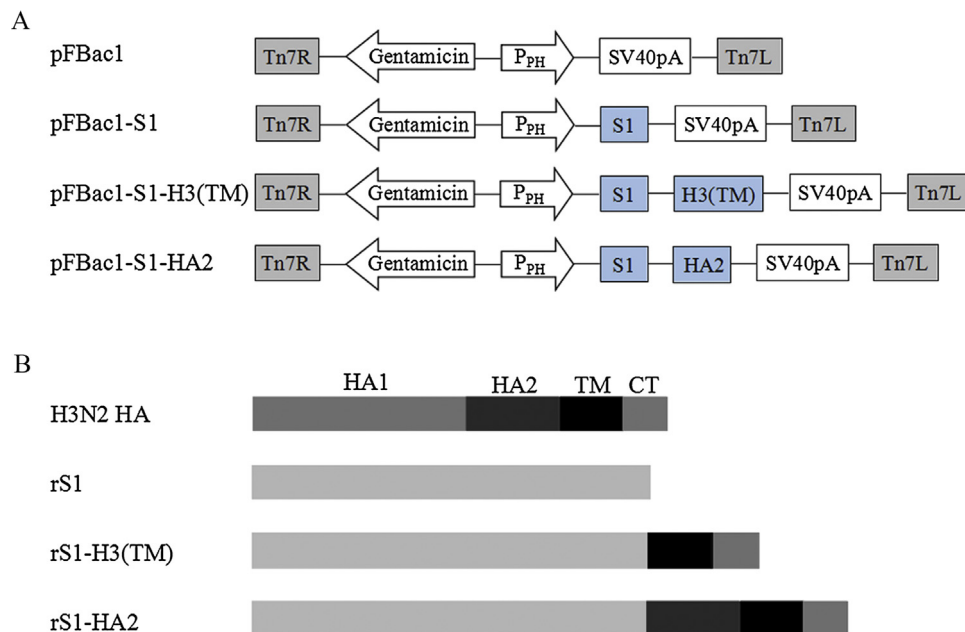
### 2.8. Cell-mediated immunity

Cellular immune response was assessed by counting the numbers of various types of functional T lymphocytes in vaccinated chickens. Two weeks after the boosting immunization and prior to challenge, five chickens were selected randomly from each group and peripheral blood samples were collected from the brachial

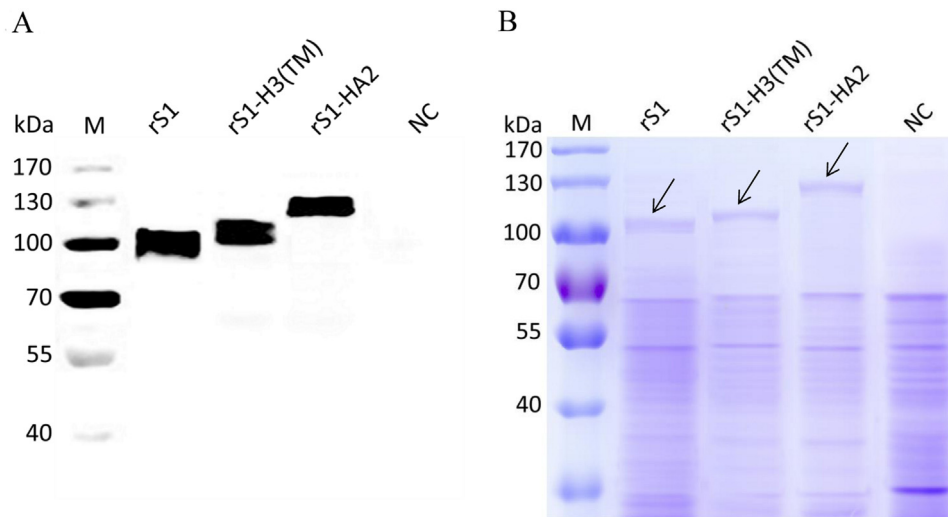
wing vein using heparinized syringes. Peripheral blood mononuclear cells (PBMCs) were isolated and adjusted to  $1 \times 10^7$  cells/ml. The samples (100  $\mu$ l;  $1 \times 10^6$  cells) were incubated for an hour at room temperature with mouse anti-chicken CD4-FITC, CD8-RPE and CD3-SPRD (Southern Biotech, Birmingham, AL, USA). Flow cytometry was performed on a FACS Calibur instrument (Becton Dickinson, San Jose, CA, USA) and data were analyzed with CellQuest software (BD Bio-sciences).

### 2.9. Detection of virus by RT-PCR

Tracheal and renal tissues of chickens were collected for detection of the challenge virus. Total RNAs were extracted from tissue suspensions using TaKaRa MiniBEST Viral RNA/DNA Extraction Kit (TAKARA Biotechnology, Dalian, China) and subjected to RT-PCR (PrimeScrip™ RT-PCR Kit, Takara Bio, China) using primers directed at the nucleocapsid as previously described (Zhang et al., 2014). The absence of detectable virus in the tracheal and kidney was considered to have been protected by the vaccine against the challenge.



**Fig. 1.** Baculovirus construct for production of proteins. (A) Construction of recombinant plasmids. pFBac1 contains polyhedrin promoter (P<sub>PH</sub>), SV40 pA, SV40 polyadenylation signal; gentamicin; Tn7R and Tn7L. Different target genes (S1, S1-H3 and S1-HA2) were inserted between P<sub>PH</sub> and SV40 pA respectively. (B) Construction of recombinant fusion proteins rS1, rS1-H3(TM) and rS1-HA2. For rS1-H3(TM) protein, rS1 protein was fused to the CT and TM domain of H3N2 HA protein; for rS1-HA2 protein, rS1 protein was fused to the HA2 domain of the HA protein of H3N2.



**Fig. 2.** Analyses of protein expression of recombinant rS1, rS1-H3(TM), and rS1-HA2 in Sf9 cells. Sf9 cells were infected with recombinant baculoviruses (rBVs). Sf9 cells infected with wild type baculoviruses were used as a negative control (NC). (A) Western blot of recombinant rS1, rS1-H3(TM) and rS1-HA2 proteins. (B) SDS-PAGE of recombinant rS1, rS1-H3(TM) and rS1-HA2 proteins. The expected bands of rS1, rS1-H3(TM), and rS1-HA2 are indicated with arrow.

### 2.10. Statistical analysis

The data were statistically analyzed by using a Student's two-tailed *t*-test when only two groups were compared or by one-way analysis of variance (ANOVA) when more than two groups were compared. *P* values (*P*) less than 0.05 were considered statistically significant.

## 3. Results

### 3.1. Expression of fusion proteins in Sf9 cells

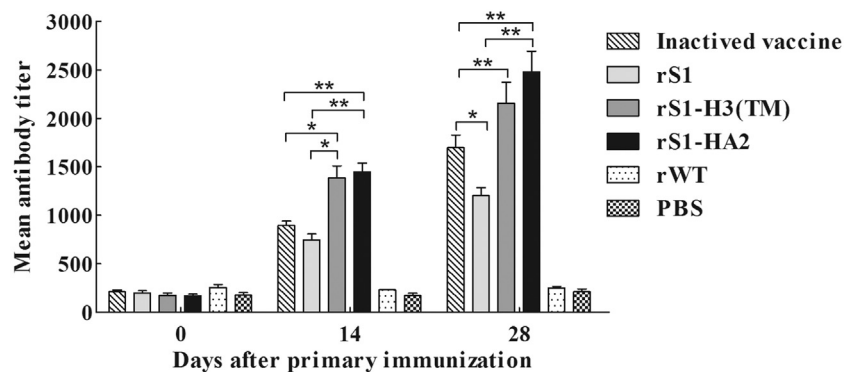
Different recombinant baculoviruses were constructed to express the target fused protein (Fig. 1A). To construct rS1-H3(TM) protein, recombinant S1 protein was fused to the cytoplasmic tail (CT) and transmembrane (TM) domain of hemagglutinin (HA) from influenza H3N2 subtype. For rS1-HA2 protein, rS1 protein was fused to the HA2 domain of the HA protein (Fig. 1B).

To check whether the three recombinant proteins rS1, rS1-H3(TM), rS1-HA2 could be expressed in the Sf9 cells, we analyzed protein expression in collected cell lysates from the recombinant baculovirus-infected Sf9 cells. Western blot under denaturing condition showed that these recombinant proteins were detectable as clearer distinct bands with molecular weight of 100 kDa, 110 kDa

and 130 kDa (Fig. 2A). The molecular weight of the recombinant rS1 protein was comparable to the native protein of 100 kDa (Cavanagh et al., 1986a,b). This result was further confirmed by SDS-PAGE using concentrated recombinant protein contents utilizing discontinuous sucrose gradient centrifugation (Fig. 2B), and these concentrated recombinant proteins were prepared for vaccination later in our study.

### 3.2. Induction of the IBV antibody response

To investigate whether the recombinant proteins could induce better immune response against IBV infection, we detected IBV-specific antibody in the sera of vaccinated chicken using indirect ELISA. In general, two weeks after primary vaccination (day 14), IBV-specific antibody level increased in all groups besides rWT and PBS. The antibody level in chickens immunized with rS1-H3(TM) and rS1-HA2 increased significantly, comparing to the rS1 group and inactivated M41 group (Fig. 3). Two weeks after booster vaccination (day 28), the levels of anti-IBV antibodies increased further in chickens immunized with inactivated M41 virus, rS1, rS1-H3(TM) and rS1-HA2. The inactivated M41 group developed a higher level of antibody than the rS1 group ( $P < 0.05$ ). Moreover, chickens vaccinated with rS1-H3(TM), rS1-HA2 had significantly higher antibody titers than animals in the rS1 group ( $P < 0.01$ ), while



**Fig. 3.** Infectious bronchitis virus (IBV)-specific antibody titers in chickens sera. Six groups of chickens ( $n = 15$  chickens per group) were subcutaneously immunized with rS1, rS1-H3(TM), or rS1-HA2, respectively. Inactivated M41 was used as a positive control, rWT (Sf9 cell lysate infected by wild-type baculoviruses) and PBS were used as negative controls. Sera from each chicken were collected and the IBV-specific antibody titers were determined by ELISA. Data is represented by the mean antibody titer +S.D.



**Table 2**  
Ratio of CD3<sup>+</sup>CD8<sup>+</sup>/CD3<sup>+</sup>CD4<sup>+</sup> T-lymphocytes 2 weeks after booster immunization<sup>a</sup>.

Group	Ratio of CD3 <sup>+</sup> CD8 <sup>+</sup> /CD3 <sup>+</sup> CD4 <sup>+</sup> T-lymphocytes
Inactivated vaccine	0.46 ± 0.10a
rS1	0.48 ± 0.05a
rS1-H3(TM)	0.71 ± 0.03b
rS1-HA2	0.73 ± 0.02b

The difference between groups with the same letter (a or b) was not significant ( $P > 0.05$ ).

<sup>a</sup> The test was repeated five times in each group. Data is presented by means ± SD.

there was no difference between the groups of rS1-H3(TM) and rS1-HA2. These data indicate that the fused S1 proteins can boost better immune responses than inactivated M41 virus and S1 protein alone, resulting in a significantly increased antibody level.

### 3.3. Cellular immune responses in vaccinated chickens

As CD3<sup>+</sup>CD4<sup>+</sup> helper T cells and CD3<sup>+</sup>CD8<sup>+</sup> cytotoxic T cells function as crucial components in cellular immunity, we counted the number of CD3<sup>+</sup>, CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocytes utilizing flow cytometry 14 days after the booster to measure immune response. Levels of CD3<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocyte increased significantly in group of rS1-HA2 and rS1-H3(TM) comparing to those groups inoculated with rS1, inactivated M41, rWT and PBS ( $P < 0.01$ ). There was no significant difference between the rS1-H3(TM) and rS1-HA2 groups. And the percentages of the T lymphocytes were comparable between the rS1 and inactivated M41 vaccine groups (Fig. 4). Further, the ratio of CD3<sup>+</sup>CD8<sup>+</sup> to CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes in rS1-H3(TM) ( $0.71 \pm 0.03$ ) and rS1-HA2 ( $0.73 \pm 0.02$ ) vaccinated groups were significantly higher than that in chickens immunized with rS1 ( $0.48 \pm 0.05$ ) and inactivated M41 ( $0.46 \pm 0.10$ ) ( $P < 0.05$ ) (Table 2). These results suggest that though rS1 alone can elicit cellular immune responses in chickens, the recombinant rS1-H3(TM) and rS1-HA2 can significantly enhance the cell-mediated immune response.

### 3.4. Protection against IBV challenge

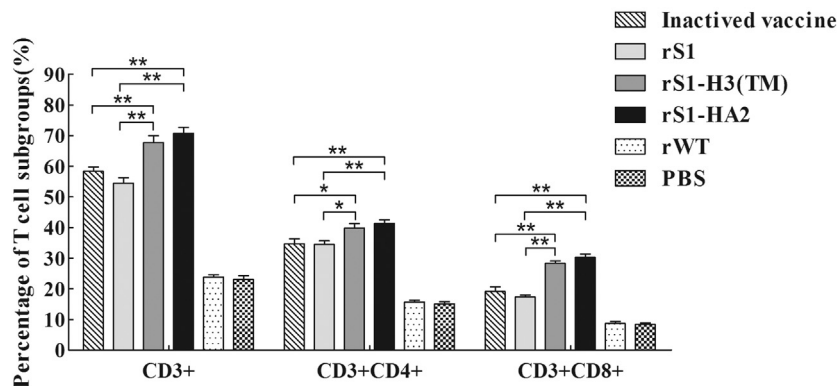
To further confirm our results, chickens were challenged with the virulent M41 strain. Morbidity, tracheal infections, renal infections and protection rates after challenge are summarized in Table 3. In general, chickens immunized with inactivated M41 vaccine, rS1, rWT and PBS developed symptoms as dyspnea, coughing and rales on a different level. To further evaluate the level of protection after challenge, RT-PCR was performed to detect viral RNA in the collected tracheas and kidneys. Chickens vaccinated with rS1 (47%) presented a similar protection level comparing to

chickens immunized with the inactivated M41 virus (53%). However, chickens immunized with rS1-HA2 were better protected (87%) comparing to chickens vaccinated with rS1 (47%) and inactivated M41 (53%) ( $P < 0.05$ ), while chickens injected with rS1-H3(TM) had similar level of protection (73%) comparing to chickens vaccinated with rS1 (47%) ( $P = 0.07$ ). There was a significant difference between the rS1-HA2-vaccinated group and the rS1-immunized group ( $p < 0.05$ ) in the number of collected tracheas and kidneys that were positive for the virus. These data further confirm our previous data presenting better immunogenesis of the fused S1 proteins and suggest that these fused proteins, especially rS1-HA2 protein, can provide a new strategy for IBV vaccine development.

## 4. Discussion

The oligomeric spike (S) protein is a trans-membrane glycoprotein, usually forms dimers or trimers that extend from coronavirus membranes. This protruding virion protein is considered to be the main antigen and therefore mainly used to induce immunogenicity against coronaviruses (Cavanagh, 1983; Delmas and Laude, 1990; Lewicki and Gallagher, 2002). For instance, researches on severe acute respiratory syndrome (SARS), which belongs to coronavirus, have suggested that recombinant trimeric full-length S proteins are highly immunogenic and able to induce an efficacious protective immune response (Kam et al., 2007; Zhou et al., 2006). More recently, Li et al. showed that the fused S protein with the foldon domain derived from T4 bacteriophage presented significantly greater potency in the induction of neutralizing antibodies than the S protein (Li et al., 2013). These researches have suggested that the manipulated S protein could serve as major target for immune therapy and vaccine design against coronaviruses.

Once this integral protruding membrane protein is assembled, it undergoes endoproteolysis and cleaves to two noncovalently associated S1 and S2 fragments (Cavanagh, 1983; Cavanagh et al., 1986a,b). The S1 domain contains the primary neutralizing epitopes for IBV, and thus is considered to be responsible determine its pathogenicity (Wickramasinghe et al., 2014). In 1998, Song et al. showed that recombinant S1 (rS1) glycoprotein, expressed in a baculovirus expression system, could induce protective immunity in chickens against challenge with virulent nephropathogenic IBV, though multiple inoculations were required and the rS1 protein induced only 50% protection of the kidney (Song et al., 1998). Other researchers displayed the S1 glycoprotein on the BacMam virus-based surface and presented that the BV-Dual-S1 protein elicited stronger humoral and cell-mediated immune responses (Zhang et al., 2014). Thus, enhancement of the antigenicity of the S1



**Fig. 4.** Flow cytometry analysis of T lymphocyte subsets in chickens after immunization with rS1, rS1-H3(TM), rS1-HA2, inactivated M41, rWT (Sf9 cell lysate infected by wild-type baculoviruses) and PBS. This test was performed two weeks after the boosting immunization. Data are presented by means + SD. n = 5 chickens per group.

**Table 3**  
Morbidity and protection rates of vaccinated chickens after IBV infection.

Group	Morbidity(%) <sup>a</sup>	No. of trachea affected <sup>b</sup>	No. of kidney affected <sup>c</sup>	Protection rate(%) <sup>d</sup>
Inactivated vaccine	27(4/15)	7/15	6/15	53(8/15) <sup>b</sup>
rS1	33(5/15)	8/15	7/15	47(7/15) <sup>b</sup>
rS1-H3(TM)	13(2/15)	4/15	3/15	73(11/15) <sup>b</sup>
rS1-HA2	7(1/15)	2/15	2/15	87(13/15) <sup>a</sup>
rWT	87(13/15)	15/15	12/15	0(0/15) <sup>c</sup>
PBS	93(14/15)	15/15	13/15	0(0/15) <sup>c</sup>

The protection rate among the five groups are indicated with different letters. The difference among groups with the same letter (a–c) was not significant ( $P > 0.05$ ).

<sup>a</sup> Morbidity was recorded for each day after challenge and is presented as total number of sick chickens in each group.

<sup>b</sup> Virus identified intrachea by RT-PCR detection.

<sup>c</sup> Virus identified in kidney by RT-PCR detection.

<sup>d</sup> Protection rate was determined by the number of unaffected chickens (without detection in tracheal and kidney)/total number of chickens in each group.

glycoprotein is considered to be critical for the development of effective vaccines against IBV.

Our previous research presented that various influenza H1, H5 and H9 hemagglutinins (HAs) proteins containing replaced H3-WT TM showed increased immunogenicity (Liu et al., 2014). To improve the immunogenicity and protective efficacy of IBV S1 protein, here we generated two fusion proteins rS1-H3(TM) and rS1-HA2 from S1 protein of IBV and hemagglutinin of influenza virus H3N2. After challenged with virulent IBV M41 strain, our results demonstrated that fusion proteins performed better protection compared with inactivated M41 vaccine and recombinant rS1 protein alone, while the latter groups presented similar protection ratio. Thus, an augment of the humoral and cellular immune responses was suggested to be induced by the fused rS1-H3(TM) and rS1-HA2 proteins.

People suggested that humoral immunity is critical for disease recovery and virus elimination (Cook et al., 1991). Consist with the immunity protective test, in this study, the level of specific antibodies induced by the recombinant rS1-H3(TM) and rS1-HA2 was higher than the rS1 and inactivated M41 group. One interesting thing is that, two weeks after booster vaccination (day 28), the antibody level induced by recombinant S1 protein was lower than that of inactivated virus in our study. It is possible that the inactivated M41 vaccine, which is derived from native viruses, comprised almost all the epitopes of IBV and thus presented better performance in inducing a IBV-specific antibody response after booster vaccination.

It is generally known that CD3<sup>+</sup>CD4<sup>+</sup> helper T cells and CD3<sup>+</sup>CD8<sup>+</sup> CTLs play a major role in inducing T cell-mediated cellular immunity (Doherty et al., 1997). Some reports have demonstrated that the CD8<sup>+</sup> CTL response are critical in the control of infectious bronchitis (IB) and more efficient than CD3<sup>+</sup>CD4<sup>+</sup> helper T cells in controlling respiratory virus infections (Cook et al., 1991; Thompson et al., 1997). The CD4<sup>+</sup> T-cell responses provide help for B-cells to mature and generate specific antibodies as well as directly antiviral cytokines, increase the proliferation, maturation, and functional activity of CD8<sup>+</sup> CTL (Doherty et al., 1997; Kotani et al., 2000). In this study, we found an significant increase of the CD3<sup>+</sup>CD4<sup>+</sup> and CD3<sup>+</sup>CD8<sup>+</sup> T lymphocyte subgroups in chickens immunized with rS1-H3(TM) and rS1-HA2, comparing to the rS1 and inactivated M41 vaccine groups. The ratio of CD3<sup>+</sup>CD8<sup>+</sup> to CD3<sup>+</sup>CD4<sup>+</sup> T lymphocytes in chickens immunized with rS1-H3(TM) or rS1-HA2 was greater than that in chickens inoculated with rS1 and inactivated M41 vaccine. These findings indicate the advantage of employing fused S1 protein to TMs or HA2 of HA proteins in an effectively stimulating cellular immune responses, especially in promoting the CTL response, which is critical for virus clearance and recovery of chickens from IBV infections (Collisson et al., 2000).

In summary, here we have successfully expressed two fused S1 proteins, rS1-HA2 and rS1-H3(TM) proteins, and demonstrated that these fusion proteins are superior to rS1 protein alone in terms of immunogenicity and protective efficacy. Accordingly, our results

suggest that the two recombinant fusion proteins rS1-HA2 and rS1-H3(TM) are suitable vaccine candidates for the development of vaccines against IBV.

### Acknowledgements

This work was supported by the Chinese National High-tech R&D Program (863 Program, 2011AA10A209), the industry, education and research of Guangdong province and Ministry of Education of People's Republic of China (Grant no. 2010B090301019), and the Science and Technology Program of Guangzhou, China (Grant no. 2014Y2-00003). We thank to Dr. George D. Liu and Dr. Yun Zhang for critical review and revision of the manuscript.

### References

- Abro, S.H., Renstrom, L.H., Ullman, K., Isaksson, M., Zohari, S., Jansson, D.S., Belak, S., Baule, C., 2012. Emergence of novel strains of avian infectious bronchitis virus in Sweden. *Vet. Microbiol.* 155 (2–4), 237–246.
- Bijlenga, G., Cook, J.K., Gelb, J.J., de Wit, J.J., 2004. Development and use of the H strain of avian infectious bronchitis virus from the Netherlands as a vaccine: a review. *Avian Pathol.* 33 (6), 550–557.
- Bosch, B.J., van der Zee, R., de Haan, C.A., Rottier, P.J., 2003. The coronavirus spike protein is a class I virus fusion protein: structural and functional characterization of the fusion core complex. *J. Virol.* 77 (16), 8801–8811.
- Cavanagh, D., Davis, P.J., Pappin, D.J., Binns, M.M., Bournsnel, M.E., Brown, T.D., 1986a. Coronavirus IBV: partial amino terminal sequencing of spike polypeptide S2 identifies the sequence Arg-Arg-Phe-Arg-Arg at the cleavage site of the spike precursor propolypeptide of IBV strains Beaudette and M41. *Virus Res.* 4 (2), 133–143.
- Cavanagh, D., Davis, P.J., Darbyshire, J.H., Peters, R.W., 1986b. Coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *J. Gen. Virol.* 67 (Pt. 7), 1435–1442.
- Cavanagh, D., 1983. Coronavirus IBV: structural characterization of the spike protein. *J. Gen. Virol.* 64 (Pt. 12), 2577–2583.
- Cavanagh, D., 2003. Severe acute respiratory syndrome vaccine development: experiences of vaccination against avian infectious bronchitis coronavirus. *Avian Pathol.* 32 (6), 567–582.
- Cavanagh, D., 2007. Coronavirus avian infectious bronchitis virus. *Vet. Res.* 38 (2), 281–297.
- Collisson, E.W., Pei, J., Dzielawa, J., Seo, S.H., 2000. Cytotoxic T lymphocytes are critical in the control of infectious bronchitis virus in poultry. *Dev. Comp. Immunol.* 24 (2–3), 187–200.
- Cook, J.K., Davison, T.F., Huggins, M.B., McLaughlan, P., 1991. Effect of in ovo bursectomy on the course of an infectious bronchitis virus infection in line C White Leghorn chickens. *Arch. Virol.* 118 (3–4), 225–234.
- Cook, J.K., Jackwood, M., Jones, R.C., 2012. The long view: 40 years of infectious bronchitis research. *Avian Pathol.* 41 (3), 239–250.
- Delmas, B., Laude, H., 1990. Assembly of coronavirus spike protein into trimers and its role in epitope expression. *J. Virol.* 64 (11), 5367–5375.
- Doherty, P.C., Topham, D.J., Tripp, R.A., Cardin, R.D., Brooks, J.W., Stevenson, P.G., 1997. Effector CD4<sup>+</sup> and CD8<sup>+</sup> T-cell mechanisms in the control of respiratory virus infections. *Immunol. Rev.* 159, 105–117.
- Ignjatovic, J., Galli, L., 1994. The S1 glycoprotein but not the N or M proteins of avian infectious bronchitis virus induces protection in vaccinated chickens. *Arch. Virol.* 138 (1–2), 117–134.
- Ignjatovic, J., Galli, U., 1995. Immune responses to structural proteins of avian infectious bronchitis virus. *Avian Pathol.* 24 (2), 313–332.
- Kam, Y.W., Kien, F., Roberts, A., Cheung, Y.C., Lamirande, E.W., Vogel, L., Chu, S.L., Tse, J., Guarner, J., Zaki, S.R., Subbarao, K., Peiris, M., Nal, B., Altmeyer, R., 2007.

- Antibodies against trimeric S glycoprotein protect hamsters against SARS-CoV challenge despite their capacity to mediate FcγRII-dependent entry into B cells in vitro. *Vaccine* 25 (4), 729–740.
- Kant, A., Koch, G., van Roozelaar, D.J., Kusters, J.G., Poelwijk, F.A., van der Zeijst, B.A., 1992. Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolyptide. *J. Gen. Virol.* 73 (Pt. 3), 591–596.
- Koch, G., Hartog, L., Kant, A., van Roozelaar, D.J., 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. *J. Gen. Virol.* 71 (Pt. 9), 1929–1935.
- Kong, Q., Xue, C., Ren, X., Zhang, C., Li, L., Shu, D., Bi, Y., Cao, Y., 2010. Proteomic analysis of purified coronavirus infectious bronchitis virus particles. *Proteome Sci.* 8, 29.
- Kotani, T., Wada, S., Tsukamoto, Y., Kuwamura, M., Yamate, J., Sakuma, S., 2000. Kinetics of lymphocytic subsets in chicken tracheal lesions infected with infectious bronchitis virus. *J. Vet. Med. Sci.* 62 (4), 397–401.
- Ladman, B.S., Pope, C.R., Ziegler, A.F., Swieczkowski, T., Callahan, C.J., Davison, S., Gelb, J.J., 2002. Protection of chickens after live and inactivated virus vaccination against challenge with nephropathogenic infectious bronchitis virus PA/Wolgemuth/98. *Avian Dis.* 46 (4), 938–944.
- Lewicki, D.N., Gallagher, T.M., 2002. Quaternary structure of coronavirus spikes in complex with carcinoembryonic antigen-related cell adhesion molecule cellular receptors. *J. Biol. Chem.* 277 (22), 19727–19734.
- Li, J., Ulitzky, L., Silberstein, E., Taylor, D.R., Viscidi, R., 2013. Immunogenicity and protection efficacy of monomeric and trimeric recombinant SARS coronavirus spike protein subunit vaccine candidates. *Viral Immunol.* 26 (2), 126–132.
- Liu, G., Lv, L., Yin, L., Li, X., Luo, D., Liu, K., Xue, C., Cao, Y., 2013. Assembly and immunogenicity of coronavirus-like particles carrying infectious bronchitis virus M and S proteins. *Vaccine* 31 (47), 5524–5530.
- Liu, Q., Liu, K., Xue, C., Zhou, J., Li, X., Luo, D., Zheng, J., Xu, S., Liu, G.D., Cao, Y., 2014. Recombinant influenza H1, H5 and H9 hemagglutinins containing replaced H3 hemagglutinin transmembrane domain showed enhanced heterosubtypic protection in mice. *Vaccine* 32 (25), 3041–3049.
- McKinley, E.T., Hilt, D.A., Jackwood, M.W., 2008. Avian coronavirus infectious bronchitis attenuated live vaccines undergo selection of subpopulations and mutations following vaccination. *Vaccine* 26 (10), 1274–1284.
- McKinley, E.T., Jackwood, M.W., Hilt, D.A., Kissinger, J.C., Robertson, J.S., Lemke, C., Paterson, A.H., 2011. Attenuated live vaccine usage affects accurate measures of virus diversity and mutation rates in avian coronavirus infectious bronchitis virus. *Virus Res.* 158 (1–2), 225–234.
- Raj, G.D., Jones, R.C., 1997. Infectious bronchitis virus: immunopathogenesis of infection in the chicken. *Avian Pathol.* 26 (4), 677–706.
- Reed, L.J., Muench, H., 1938. A simple method of estimating fifty percent endpoints. *Am. J. Hyg.* 27, 493–497.
- Schultze, B., Cavanagh, D., Herrler, G., 1992. Neuraminidase treatment of avian infectious bronchitis coronavirus reveals a hemagglutinating activity that is dependent on sialic acid-containing receptors on erythrocytes. *Virology* 189 (2), 792–794.
- Sjaak, D.W.J., Cook, J.K., van der Heijden, H.M., 2011. Infectious bronchitis virus variants: a review of the history, current situation and control measures. *Avian Pathol.* 40 (3), 223–235.
- Song, C.S., Lee, Y.J., Lee, C.W., Sung, H.W., Kim, J.H., Mo, I.P., Izumiya, Y., Jang, H.K., Mikami, T., 1998. Induction of protective immunity in chickens vaccinated with infectious bronchitis virus S1 glycoprotein expressed by a recombinant baculovirus. *J. Gen. Virol.* 79 (Pt. 4), 719–723.
- Thompson, G., Mohammed, H., Bauman, B., Naqi, S., 1997. Systemic and local antibody responses to infectious bronchitis virus in chickens inoculated with infectious bursal disease virus and control chickens. *Avian Dis.* 41 (3), 519–527.
- Wickramasinghe, I.N., van Beurden, S.J., Weerts, E.A., Verheije, M.H., 2014. The avian coronavirus spike protein. *Virus Res.* 194, 37–48.
- Zhang, J., Chen, X.W., Tong, T.Z., Ye, Y., Liao, M., Fan, H.Y., 2014. BacMam virus-based surface display of the infectious bronchitis virus (IBV) S1 glycoprotein confers strong protection against virulent IBV challenge in chickens. *Vaccine* 32 (6), 664–670.
- Zhou, Z., Post, P., Chubet, R., Holtz, K., McPherson, C., Petric, M., Cox, M., 2006. A recombinant baculovirus-expressed S glycoprotein vaccine elicits high titers of SARS-associated coronavirus (SARS-CoV) neutralizing antibodies in mice. *Vaccine* 24 (17), 3624–3631.