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

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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.

1. Introduction

Avain 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

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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_{50} 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 SalI and HindIII sites of pFastBac1 vector (Invitrogen) to generate recombinant shuttle plasmids (Fig. 1A). The shuttle vectors were then chemically transformed into competent DH10Bac™ 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™ 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 2×10^4 EID_{50} 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 euthanised 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.