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The immunoreactivity of a chimeric multi-epitope DNA vaccine against IBV in chickens

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ABSTRACT

Epitope-based vaccines designed to induce cellular immune response and antibody responses specific for infectious bronchitis virus (IBV) are being developed as a means for increasing vaccine potency. In this study, we selected seven epitopes from the spike (S1), spike (S2), and nucleocapsid (N) protein and constructed a multi-epitope DNA vaccine. The 7-day-old chickens were immunized intramuscularly with multi-epitope DNA vaccine encapsulated by liposome and boosted two weeks later, and were challenged by virulent IBV strain five weeks post booster. The results showed that multi-epitope DNA vaccine led to a dramatic augmentation of humoral and cellular responses, and provided up to 80.0% rate of immune protection. The novel immunogenic chimeric multi-epitope DNA vaccine revealed in this study provided a new candidate target for IBV vaccine development.

Infectious bronchitis virus (IBV) is one of the primary causes of respiratory disease in domestic fowl. Infection with IBV reduces the performance of broilers and in laying birds drops in egg production and egg quality can occur [1]. Vaccines against infectious bronchitis applied in the field have respective weakness. Live attenuated vaccine has a strong probability of spreading of the vaccine virus [2]; inactivated vaccine does not elicit enough immune response against coronavirus [3,4], and shows severe side effect and long-lasting local response after inoculation. The construction of conventional DNA vaccine has to be based on integral antigen gene, and only one or two gene at the most can be constructed into one plasmid, so its immune protection is imperfect. However, epitope-based DNA vaccine breaks through such weakness of traditional DNA vaccine. It can not only elicit favourable immunoreactivity, but also has no risk of gene integrating into host cell [5].

The IBV encodes four major structural proteins, known as spike (S) protein, nucleocapsid (N) protein, membrane (M) protein, and envelope (E) protein. The S protein is post-translationally cleaved into the outer S1 and the membrane bound S2 proteins. S1 protein comprises major antigenic determinants that induce neutralizing Abs which makes it a major target for vaccine design and immune therapy [6–9]. S2 protein is conserved and comprises epitopes inducing cross-reactive Abs and cell-mediated immune (CMI) responses. N protein is also largely conserved, and contains epitopes which induce cytotoxic T lymphocyte (CTL) responses and also protection, as well as activating B cell responses [10–12].

In this paper, seven dominant epitopes corresponding to four regions in the S1, one in the S2, and two in the N protein were selected according to previous references as well as computer bioinformatics software. The selected epitopes contained both B-cell epitopes that were essential for protective antibody response and T-cell epitopes that would serve to induce CTL response. The corresponding epitope minigenes were combined together and constructed as a multi-epitope chimeric DNA vaccine, then we investigated the cellular and humoral immunity induced by the multi-epitope vaccine and its protective effect in chickens.

Materials and methods

Virus, experimental animals, and cells. The specific-pathogen-free (SPF) chicken embryos were purchased from Shangdong Institute of Poultry Science, Shandong, PR China. Chickens were hatched and housed in a SPF environment at the Laboratory Animal and Resources Facility, Sichuan University. The nephro-pathogenic IBV SAIBk strain was propagated in the allantoic cavities of 10-day-old SPF embryonated chicken eggs, and then harvested allantoic fluid 36 h post-inoculation. The 50% chicken infection dose (EID_{50}) was determined by inoculating serial 10-fold dilutions of virus into 10-day-old SPF embryonated chicken eggs. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin at pH 7.2 and were kept at 37 °C with 5% carbon dioxide.

Multi-epitope genes selection. The nucleinic acid sequences of S1, S2 and N gene of the IBV SAIBk strain were obtained from GenBank

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(Accession No. DQ288927). The epitope sequences of the three genes were analyzed according to ExPasy network software (http://www.ExPasy.org) and reported references, and then seven T- and B-cell epitopes from S1, S2, and N protein (S1: 24-150,240-255,290-400,532-537; S2:1-65; N:1-120, 290-410) were selected (Table 1).

Design and construction of the multi-epitope chimeric DNA vaccine. The seven multi-epitope minigenes were paiallelled as a single chimeric gene separated from each other with the GA/GP spacers with an unique open reading frame by using Splicing by Overlap Extension (SOEing) and polymerase chain reaction (PCR) (Table 1). The constructs included a Kozak sequence at the N-terminus and CpG motif in the middle of the chimeric gene to enhance immune response. The multi-epitope chimeric gene was then incorporated into expression vector pVAX1 (Invitrogen, USA) designated as pVAX-F, which was confirmed by endonuclease digestion assay and DNA sequencing.

Indirect immunofluorescence analysis. Six-well tissue culture plates were seeded with COS-7 cells (10^6/well). Monolayer of 80–90% confluent cells was transiently transfected with the plasmid pVAX-F and empty plasmid by using Lipofactamin Reagent (Invitrogen, CA, USA). Thirty-six hours after transfection, cells were washed with phosphate-buffered-saline (PBS), fixed with 100% acetone for 10 min at −20 °C and washed once again with PBS. Diluted primary and secondary antibodies were incubated at 37 °C for 1 h, respectively. Primary antibodies used were antiserum of rabbit to IBV, and secondary antibodies were FITC-conjugated–goat- anti-rabbit IgG (Sigma).

Immunization of chickens with plasmid DNA vaccine. The plasmids pVAX-F and pVAX1 amplified in *Escherichia coli* DH5α were extracted using the alkaline lysis method, then purified by PEG8000 precipitation. For DNA immunizations, chickens at 7-day-old were randomly divided into four groups (n = 15 each).The chickens were immunized intramuscularly with 150 μg of plasmid pVAX-F(group 1), and 150 μg of empty plasmid pVAX1 (group 2). Another group administered with 0.5 ml of IBV inactive vaccine (group 3), and chickens injected with 0.5 ml PBS as control (group 4). All groups were boosted with an equivalent dose at 14 days after the initial inoculation. For DNA immunizations, chickens at 7-day-old were randomly divided into four groups (n = 15 each).

Detection of anti-IBV specific antibodies. Sera were collected after booster vaccination until challenge, and pre-vaccination sera were also collected. Total serum immunoglobulinG (IgG) specific for IBV was measured by indirect enzyme-linked immunosorbent assay (ELISA). The test sera were diluted by 1:500 and then manipulated according to the instruction of IBV antibody detection ELISA kit (IDEXX, USA), and the optical density at 650 nm was measured in an ELISA microplate reader. Every test serum was run in triplicate in each assay, as well as including negative and positive control sera.

Analysis of CD4+ and CD8+ T-lymphocytes. Peripheral blood samples from immunized chickens were collected from the wing vein 7 days after the booster. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll–Hypaque density gradient centrifugation and adjusted to 1×10^7 cells/ml. One-hundred microliters of cell suspensions (1×10^6 cells) was incubated for 1 h at room temperature with both mouse anti-chicken CD4-PE and mouse anti-chicken CD8-FITC (BD Biosciences Pharmingen) simultaneously. The samples were processed on fluorescence activated cell sorter.

Protection against virulent challenge. All of the chickens were challenged with $100EID_{50}$ of the IBV SAIBk strain in 0.1 ml by the nasal-ocular route at 35 days after the booster, and were examined daily for 2 weeks for the clinical symptoms such as coughing, sneezing, ataxia, dyspnea or death. Dead chicks were necropsied to confirm the death due to IBV infection. Chickens in each group were euthanized at 14 days post challenge, then necropsies were performed immediately and kidney tissues were collected for further detection of virus by RT-PCR.

Results

Construction of chimeric multi-epitope DNA vaccine

The full length of the constructed multi-epitope chimeric gene 'F' was 1743 bp. The chimeric gene was then incorporated into a pVAX1 vector, and the recombination plasmid with the correct insert orientation designated as pVAX-F was detected by sequencing (data not shown), restriction analysis and PCR (Fig. 1A).

Expression of recombinant plasmids in COS-7 cells

The expression of pVAX-F was demonstrated by indirect immunofluorescence assay. The cells transfected by pVAX-F displayed fluorescence in cytoplasm (Fig. 1B), which showed constructs encoding chimeric multi-epitope F protein can be expressed successfully in the eukaryotic system.

Humoral immune reaction induced by DNA vaccine

The plasmid pVAX-F, inactive vaccine induced significantly increased antibodies to IBV Ag in chickens one week after booster, respectively, while there were no detectable specific antibodies in the plasmid pVAX1 group and PBS group. And the

Table 1
Character of each selected epitope and used primers in the design.

Epitope	Origin	Position(aa)	Epitope type	Synthesized epitope genes and used primers(5′-3′) in PCR[a]
F1	S1	24–150	B (neutrallization)	S: GTA *GGATCC* CCACCATGG ATAATTATGTG A: TCA *GGGCCC* ATTATAAAATAGA
F2	S1	240–255	B	5′ CAATACTGGTAATTTTTCAGATGGGTTTTACC CTTTTACTAATTCTAGTGGCGC 3′
F3	S1	290–400	B (neutrallization)	S: ACT *GGCGCC* CAAGGTGGTGTTCA A: AGT *GGGCCC* TTCAAAATTTTGTC
F4	S1	532–537	T	5′ CATTAAACTCACTAAAGAGGGT **AACGTT** GGCGC 3′
F5	S2	1–65	B (neutralization)	S: ACT *GGCGCC* TCTACTAGTGAAAATG T(CTL) A: AGT *GGGCCC* ACGTGTTTGTATGTA
F6	N	1–120	T (CTL)	S: AGT *GGGCCC* GCAAGCAGTAAGGCA B A: ACT *GGCGCC* ACCCTTAGCAGCA
F7	N	290–410	T (CTL)	S: AGT *GGCGCC* AAGCTTCAACCTGA A: ACT *CTCGAG* TTATTAGAGTTCATTTTC

[a] *Note.* Letters in italic means restriction sites, underlined means Kozak sequence, bloded means CpG motif. 'S' means sense primer, while 'A' means anti-sense primer.

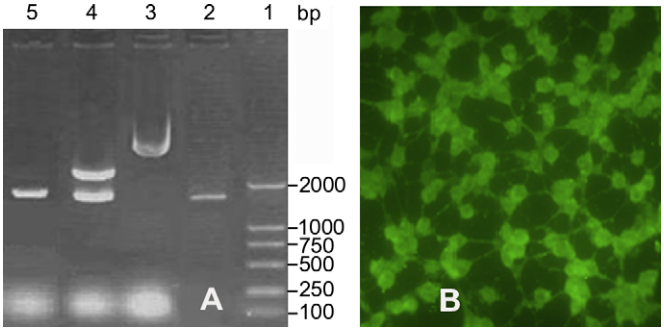

Fig. 1. Identification of plasmid pVAX-F. (A) Chimeric gene F and verification of plasmid pVAX-F. Lane 1, DL2000; lane 2, constructed chimeric gene F was 1743 bp; lane 3, plasmid pVAX-F; lane 4, pVAX-F digested by EcoRI and XbaI; lane 5, chimeric gene F amplified by PCR from pVAX-F. (B) Indirect immunofluorescence detection of the expressed chimeric protein in COS-7 cell. Cells transfected with plasmid pVAX-F showed positive results.

IBV antibody titer of chimeric multi-epitope DNA vaccine immunized group was higher than that of inactive vaccine group (Fig. 2A).

Cellular immune reaction induced by DNA vaccine

Peripheral blood lymphocytes were analyzed by flow cytometry 7 days after the booster. The percentage of CD4+CD3+ and the percentage of CD8+CD3+ T-lymphocytes in pVAX-F vaccinated group were significantly higher ($P < 0.05$) than those of the pVAX1 group and PBS group, respectively, and also higher than that of inactive vaccine group (Fig. 2B). The percentage of the two T-lymphocyte subgroups had no significant difference between pVAX1 and PBS group, respectively.

Protection after challenge

The mortality rates and protection rates of different groups in the challenge trial were summarized in Table 2. Chickens started

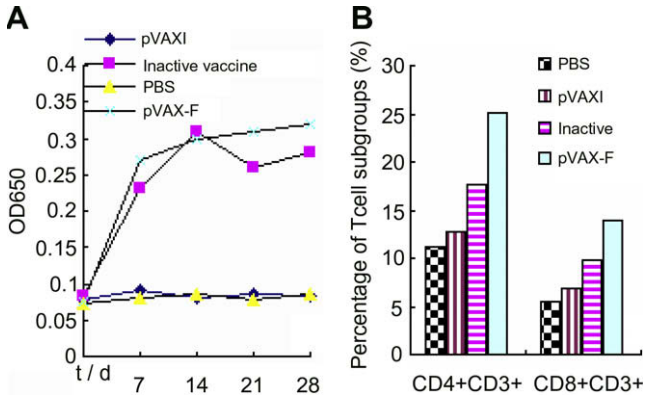

Fig. 2. Humoral and cellular responses in immunized chickens. (A) Peripheral blood anti-IBV ELISA antibody levels of immunized chickens. Sera from all of chickens were sampled weekly post booster. The result was obtained from average of five sera in each group every assay. The data of antibody titers was analyzed by software of Statistics Package for Social Science (SPSS). The results showed that the antibody titers of multi-epitope DNA vaccine-immunized group on day 7(0.270 ± 0.020), 21(0.310 ± 0.040), 28(0.320 ± 0.020) post booster immunization were all significantly higher than that of inactive vaccine group(0.230 ± 0.030; 0.260 ± 0.010; 0.280 ± 0.005), respectively ($P < 0.05$). Though the antibody titer of multi-epitope DNA vaccine group on day 14(0.300 ± 0.010) was lower that of inactive vaccine group(0.310 ± 0.020), there was no significant differences between the two groups. (B) The percentage of CD4+CD3+ and CD8+CD3+ T-lymphocytes of different inoculated groups. This test was performed 7 days after boosting immunization.

to show clinical signs or die from viral infection on day 6 after challenge. The chickens immunized with either empty vector pVAX1 or PBS were not protected and developed coughing, nasal discharge and dyspnea. The death rate of the pVAX1 and PBS injected groups was 40.0% and 46.67% at 14 days post challenge, respectively. The death rate of the pVAX-F DNA vaccine immunized chickens was only 6.67%, lower than 13.33% of the inactive vaccine immunized chickens. Moreover, the clinical signs and pathological changes of the affected chickens in the inactive vaccine group were more conspicuous and severe than that of affected chickens in pVAX-F immunized group. To evaluate the level of response after challenge, the collected kidney samples were detected by RT-PCR for IBV infection. PCR results indicated that 20.0% and 26.67% of chickens vaccinated with plasmid pVAX-F and inactive vaccine were positive for the presence of virus in the kidney, respectively. All chickens immunized with either empty vector pVAX1 or PBS were positive in RT-PCR test. The group vaccinated with pVAX-F DNA vaccine had the highest rate of protection (80%) in all vaccinated groups. This suggests that the constructed multi-epitope chimeric DNA vaccine offers considerable resistance against a virulent IBV challenge.

4. Discussion

Multi-epitope DNA vaccine, which is a newly-developed DNA vaccine based on both traditional DNA vaccine and polypeptide vaccine, was constructed by significant multi-epitope genes and separated one from another with appropriate spacers [13–15]. The essence of this technology is that short DNA sequences, encoding well-defined cytotoxic T-lymphocyte (CTL), antibody or helper T-lymphocyte (HTL) specific epitopes are used as immunogens, which is often capable of inducing protection against a large and complex pathogen. Compared to other vaccine strategies, several potential advantages are apparent. First, epitope-based DNA vaccine strategy shows increased 'safety' of an immunisation strategy that mimics antigen processing and presentation during natural infections, without actually causing disease. Secondly, it has the 'flexibility' in epitope selection, which allows induction and optimisation of the desired type of immunity. In addition, the epitope-based constructs appear to be capable of inducing more potent immunoreaction than whole protein vaccines. Furthermore, multiple epitopes derived from more than one antigen can be packaged into a relatively small delivery vehicle, and generate broad responses simultaneously targeting multiple Ags. Finally, epitope-based vaccines may overcome immunodominance and T cell tolerance. Therefore, this paper presented and discussed the construct of chimeric multi-epitope DNA vaccine of IBV and its immune response in chickens. The preclinical studies available to date clearly demonstrated the potential of this vaccine approach in terms of prophylaxis.

For the structural proteins of IBV, the S1 glycoprotein induces protection against virulent challenge [16,17], and several antigenic sites that induce virus neutralising antibodies have been mapped on S1 protein [18,19]. The S2 glycoprotein induces cross-reactive ELISA antibodies, and it is not involved in protection [16], but it was shown in one study that one region located in the S2 may have a role in protection [10]. The N protein is highly conserved, highly immunogenic. It induces cross-reactive antibodies and is the most abundant virus-derived protein produced throughout infection [11]. N protein may also induce protective immunity [20]. Moreover, epitopes had been mapped in N protein [11,21], and one region was identified that induces a T-cell response and also protection [12]. Therefore, the seven selected dominant epitopes in this study contained CTL epitopes and neutralizing B-cell epi-

Table 2
The mortality and protection rate of different groups challenged by virulent IBV SAIBk strain.

Groups	No. of death	No. of affected[a]	Mortality[b] (%)	Protection rate[c] (%)
pVAX-F	1	3	6.67	80.00
Inactivated vaccine	2	4	13.33	73.33
pVAX1	6	15	40.00	0
PBS	7	15	46.67	0

[a] Affected was determined by RT-PCR positive birds from dead and euthanized chickens' kidneys.
[b] Mortality was recorded for each day after challenge and is presented as total number of dead chickens in each group.
[c] Percent of protection was determined by the number of unaffected chickens/total number of chickens in each group. It was showed that 12 out 15 chickens in the multi-epitope DNA vaccine immunized group didn't affect IBV, and 11 out of 15 chickens in was protected in inactive vaccine group, while all the chickens in pVAX1 group and PBS group were affected IBV.

topes so as to elicit protective immune responses against virulent IBV.

In the present study, several strategies had been adopted when we constructed the multi-epitope DNA vaccine to enhance the protective effect of epitope-based gene immunization. First, the spacers between two authentic epitopes were usually designed as flexibility peptide [22] or prolease recognizing sequence [23] to eliminate interference of two consecutive epitopes. In this study we constructed the GA/GP as spacer between two epitopes which not only minimized junctional epitopes, but also simultaneously optimized for proteosome processing. Secondly, CpG motif in plasmid as well as CpG oligodeoxynucleotides (ODN) can activate systemic and mucosal immune responses, induce lymphocytes and macrophages to secrete polyreactive antibodies and/or cytokines, and induce proliferation of B-cell [24–27]. We inserted CpG motif in the multi-epitope chimeric gene to enhance immune response. In addition, the Kozak sequence was also constructed at the 5′ terminal of chimeric gene to improve the expression according to previous study [28].

In this paper, the level of specific antibodies developed in pVAX-F group was higher than that of pVAX1 group and PBS group, respectively, and also superior than inactive vaccine group, which suggested the DNA vaccine induced remarkable humoral response. Studies demonstrated that humoral immunity plays an important role in disease recovery and virus clearance [29,30]. In addition, it is also reported T-cell response played an important role in efficacy protection [6,12,31].

Results of T-lymphocytes subgroup detection indicated, that the percentage numbers of CD4+CD3+ and CD8+CD3+ T-lymphocytes subgroups in pVAX-F vaccinated chickens group were higher than those in the pVAX1 vaccinated chickens. This demonstrated that the constructed multi-epitope chimeric DNA vaccine stimulated intensive cellular immunoreaction. Since CD8+ CTL are critical in the control of infectious bronchitis in poultry, and CD4+ T-cell responses may increase the proliferation, maturation and functional activity of CD8+ CTL, providing increased help for B-cells and directly producing antiviral cytokines, the abundantly increased T-lymphocytes in the pVAX-F group may provide a better efficiency of protection.

The virus challenge assay showed chickens received pVAX-F were better protected than inactive vaccine group, as well as control groups certainly. The protection rate of pVAX-F group was up to 80.0%, possibly indicating evident protective immunity overcome by virus aggressiveness.

Results of immune response and viral challenge showed the group inoculated with pVAX-F provided strong immune response and remarkable protection rate. These results indicated the constructed multi-epitope chimeric DNA vaccine is a feasible approach to provide protection against IBV challenge.

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