FULL PAPER *Immunology*

Multivalent DNA Vaccine Enhanced Protection Efficacy against Infectious Bronchitis Virus in Chickens

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ABSTRACT. For efficacious DNA vaccine development against infectious bronchitis virus (IBV), the immunogenicity of a multivalent DNA vaccine was evaluated. Three expression plasmids each targeting spike protein (S1), nucleocapsid protein (N), and membrane protein (M) of IBV were prepared. Chickens were immunized with either individual plasmids (monovalent) or with a combination of all plasmids (multivalent). Immunization with the multivalent DNA vaccine induced synergistic augmentation of humoral and cellular responses in comparison with the individual vaccines, and provided up to 85% immune protection. Thus the multivalent DNA vaccine represents an innovative approach for enhancing DNA vaccine potency, and has potential clinical application for vaccination against IBV.
KEY WORDS: DNA vaccine, infectious bronchitis virus, multivalency.

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Infectious bronchitis virus (IBV) belongs to the family *Coronaviridae*, which has the largest RNA viral genomes, ranging from 26–32 kb in length [20]. IBV can infect the trachea, kidney, and oviduct of the adult chicken resulting severe impairment of egg laying and poor egg quality [1, 8]. Given the economic effects of IBV on the commercial poultry industry, its prevention has been continually pursued. At present, control of IBV is generally achieved by vaccination with inactivated and live-attenuated vaccines. However, weaknesses have been found in both vaccines. For instance, inactivated vaccines frequently fail to induce strong cellular immunity [5], while live attenuated vaccines exhibit a strong probability of reverse to virulence via mutation, resulting in further spreading of the virus [12, 24]. As such, there is a pressing need for the development of more safe and effective vaccines.

DNA vaccines are a novel strategy used for immunization. In contrast with traditional vaccines, DNA vaccines are able to induce both cellular and humoral immune responses which produce long-lasting immunity [11, 21, 33]. In addition, DNA vaccines are easily manufactured since no live microorganisms are required, thus reducing environmental contamination [22, 29]. Like other coronaviruses, IBV contains four essential structural proteins: the spike (S), integral membrane (M), small envelope (E) membrane proteins and a phosphorylated nucleocapsid (N) protein. The S protein is post-translationally cleaved into two subunits: the outer S1 spike protein and the membrane bound S2 proteins. The S1 spike protein subunit is necessary and sufficient to induce protective immunity, and has been successfully constructed as a DNA vaccine against IBV [6, 18]. The N protein was reported to induce Cytotoxic T lymphocyte (CTL) responses which are critical in the control of IBV in poultry [7, 28], and was shown to provide certain protection against IBV as a DNA vaccine in our previous study [30]. The M glycoprotein was also shown to induce detectable antibodies and delayed type hypersensitivity (DTH) responses [13]. Hence, these proteins are primary targets for DNA vaccine development for elicitation of immune responses.

Recently, it has been demonstrated that vaccination with a DNA vaccine combination can protect mice against *M. tuberculosis* infection and poxvirus challenge more effectively than vaccination with either gene alone [3, 15, 16]. Luo and his research group had detected markedly increase of humoral and cellular immune responses elicited by a divalent fusion DNA vaccine indicating a broader antigen repertoire may result in improved anti-virus activity [23]. Thus, in present study, we constructed three DNA vaccines against S1, M, and N, and applied them in combination to evaluate their protection against IBV challenge.

MATERIALS AND METHODS

Animals, viruses, and cell line: SPF chicken embryos were purchased from Beijing laboratory animal research center, China. Chickens were hatched and housed in HEPA-filtered isolators at the Laboratory Animal and Resources Facility, Sichuan University. The nephro-pathogenic IBV SAIBk strain was isolated from a field outbreak in Sichuan province in China [30]. The IBV SAIBk strain was propagated in 10 day old specific-pathogen-free (SPF) chicken embryos, and allantoic fluid was then collected for viral isolation. Vero cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen Corporation, Carlsbad, CA, U.S.A.) supplemented with 10% fetal bovine serum (FBS), 100 U/m*l* penicillin, and 100 μg/m*l* streptomycin

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(pH 7.2), and were maintained at 37°C with 5% carbon dioxide.

Construction of recombinant plasmid: The cDNA sequences of S1, M, and N genes of the IBV SAIBk strain were obtained from GenBank (GenBank accession no. DQ288927). The full-length cDNA sequences of the three genes were amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using the following primers: S1 gene, 5'-CCC*AAGCTT*ACCATGGTGGGGAAGT-CACTG-3' (upstream primer with *Hin*dIII site underlined), 5'-CGCGGATCCTTACATTTTCACTAGTAGAACGT-3' (downstream primer with *Bam*HI site underlined); M gene, 5'-CGC*GGATCCACC*ATGGCCAACGAGACA-3 (upstream primer with *Bam*HI site underlined), 5'-CCG-GAATTCTTATGTGTAAAGACTACCT-3' (downstream primer with EcoRI site underlined); and N gene, 5'-CGCG-GATCCACCATGGCAAGCGGTAAGGCAAC-3' (upstream primer with *Bam*HI site underlined), 5'-CCG-*GAATTC*TCAGAGTTCATTCTCACCT (downstream primer with *Eco*RI site underlined). The full-length S1, M, and N genes were first cloned into TOPO pCR™ 2.1 vectors (Invitrogen Corporation) by TA cloning, and then subcloned into the expression vector pVAX1 (Invitrogen Corporation). All the constructs were confirmed by sequence analysis.

In vitro expression of the plasmid DNA: Monolayers of Vero cells were transfected using Lipofectamine 2000 reagent (Invitrogen Corporation) following the manufacturer's instructions. In brief, 300 g of DNA was diluted into 50 μl medium without serum. Next, 3 μl of Lipofectamine™ 2000 was added into 50 μl medium. The cells were then transfected with DNA-Lipofectamine™ 2000 complexes (100 μl). Thirty-six hours after transfection, the cells were fixed with 4% paraformaldehyde. Next, the cells were incubated in 2% BSA in PBS (0.02% Tween 20) to block nonspecific reactive sites, and then incubated overnight at 4°C with rabbit anti-IBV polyclonal antisera. The bound antibody was detected after a further incubation with FITC-conjugated goat anti-rabbit IgG (Sigma-Aldrich, St. Louis, MO, U.S.A.) that included 0.05% azovan blue (to identify positive and negative Vero cells). The Vero cells with S1, M, and N protein expression were stained green while the Vero cells with no S1, M, and N protein expression were stained red.

Immunization of chickens with DNA vaccines: Standard large-scale extraction and purification of the plasmids were performed, as previously described [25]. The dehydration and rehydration procedure was used for the incorporation of the DNA vaccine into cationic liposomes [14, 31]. The seven day old SPF chickens were then randomly divided into six groups with 20 chickens per group. Groups 1–3 received 100 μg of pVAX1-S1, PVAX1-M, or pVAX1-N, respectively; Group 4 received 100 μg combined DNA vaccine containing 33 μg of each of the three antigen plasmids; Groups 5–6 received 100 μg empty pVAX1 and 0.5 m*l* PBS. All the chickens were immunized intramuscularly with the vaccines at seven days of age. After two weeks, the chickens were boosted with the same DNA vaccines (same route and dosage).

Antibody titers to IBV by enzyme-linked immunosorbent assay (ELISA): Blood was collected from the wing vein (500 μl) of chickens at 7, 14, 21, 28, and 35 days of age. The test sera were diluted 1:500 and then manipulated according to the instruction of a commercial antibody capture ELISA kit for IBV (IDEXX, MA, U.S.A.). The relative level of antibody titer was determined by calculating the sample to positive (S/P) ratio as [(mean of sample optical density)-(mean of negative control optical density)]/[(mean of positive control optical density)-(mean of negative control optical density)]. Endpoint titers were calculated with the equation: log10titer=1.09 (log10 S/P) + 3.36 (FlockCheck program, IDEXX). As recommended by the supplier, titers greater than log10 2.60 which is equal to 396 were considered positive. Group mean titers were calculated from individual log10 titers.

$CD4^+$ and $CD8^+$ positive T lymphocytes assay: Blood was collected from the jugular vein of the immunized chickens in heparinized capillary tubes to prevent clotting. Peripheral blood mononuclear cells (PBMCs) were isolated from each blood sample by Ficoll-Hypaque density gradient centrifugation. PBMCs were adjusted to 1×10^7 cells/m*l*. The samples (100 μl; 1×10^6 cells) were incubated for 1 hr at room temperature with mouse anti-chicken CD4-PE (BD Biosciences, Franklin Lake, NJ, U.S.A.), mouse anti-chicken CD3-SPRD (BD Biosciences) and mouse anti-chicken CD8-FITC (BD Biosciences) primary antibodies. Thus, the Leukocyte samples were triply labeled with CD3, CD4 and CD8 antibodies. Finally, the fluorescent positive cells were analyzed with a flow cytometer (Beckman Coulter Inc., Fullerton, CA, U.S.A.).

Virus challenge: All chickens were challenged with $100EID_{50}$ of the IBV SAIBk strain in 0.1 m*l* by a nasal-ocular route at 21 days after the boost immunization. Clinical symptoms were observed daily for 14 days post-challenge. Dead chickens were necropsied to confirm death by IBV infection. The surviving chickens were euthanized at 14 days post-infection, and their kidney tissues were collected for further virus detection.

Detection of virus in kidney tissues by RT-PCR: The IBV in the kidney tissues of the challenged chickens was detected by RT-PCR, as this technique was more rapid and sensitive than virus isolation as a tool for virus detection. In brief, total RNA was extracted by using RNAprep pure tissue kit (Tiangen, China), and then subjected to RT-PCR using primers directed to the 3'-untranslated region (forward primer: 5-GATGAGGAGAGGAACAATGC-3; reverse primer: 5-TGGGCGTCCTAGTGCTGT-3). Total protection was defined as negative for the presence of virus in the kidney.

Statistical analyses: Data were analyzed using the one-sided Student's *t* test. Differences were considered statistically significant with $P<0.05$.

RESULTS

Construction of recombinant plasmids: The full length S1, M, and N genes of IBV were constructed with the expected sizes (S1: 1669 bp, M: 678 bp, N: 1230 bp; Fig. 1). Depending on the specified restriction enzyme cutting sites, these fragments were further directionally cloned into the expression vector pVAX1. Results of the restriction enzyme digestion confirmed gene insertion direction (Fig. 1).

In vitro expression of recombinant plasmids in Vero cells: To evaluate the expression levels of the recombinant plasmids, the expression constructs were transfected into Vero cells. Green fluorescence was detected in the cells transfected with pVAX1-S1, pVAX1-M, and pVAX1-N (Fig. 2). In contrast, no green fluorescence signal was detected in the control cells where the empty expression vector was transfected. These data demonstrate that the constructs encoding S1, M, and N protein were successfully expressed in the eukaryotic system.

Antibody responses in chickens induced by the plasmid vaccination: The antibody responses were detected in chickens immunized with the constructs encoding the S1, M, and N proteins. Anti-IBV antibody levels were increased in chickens immunized with either pVAX1-S1, pVAX1-M, pVAX1-N or the three constructs in combination (Fig. 3). Interestingly, a higher antibody titer level was observed in chickens immunized with the three expression constructs in combination compared with the individual constructs, suggesting a greater potency for inducing antibody response. In contrast, no anti-IBV antibody response was detected in chickens immunized with PBS or the control pVAX1 vector.

T-lymphocyte subset analysis: An effective anti-virus vaccine requires the collaboration of both immune systems, such as the induction of cellular ($CD4^+$ and $CD8^+$ T cells) as well as antibody responses [10]. In the present study, peripheral blood lymphocytes were analyzed by flow cytometry to evaluate the cellular immune responses at one week after the boosting immunization. Levels of CD4 and CD8 positive T cells were higher in the chickens immunized with the pVAX1-N construct than in those immunized with

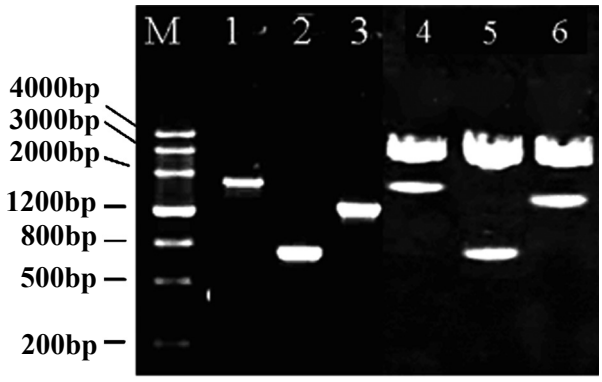

Fig. 1. Agarose gel electrophoresis of PCR products and enzyme digestion analysis. Lanes: M, DNA molecular weight markers; lane 1, S1 gene RT-PCR amplified from IBV; lane 2, M gene RT-PCR amplified from IBV; lane 3, N gene RT-PCR amplified from IBV; lane 4, plasmid pVAX1-S1 digested with *Bam*HI and *Hind*III; lane 5, plasmid pVAX1-M digested with *Bam*HI and *Eco*RI; lane 6, plasmid pVAX1-N digested with *Bam*HI and *Eco*RI.

the pVAX1-S1 or the pVAX1-M constructs ($P<0.01$; Fig. 4). As for the antibody responses described above, the highest cellular immune response was detected in chickens immunized with the three expression constructs in combination, confirming its potency against IBV. There was no significant difference between the control pVAX1 and the PBS groups for the two T-lymphocyte subgroups.

Protection against IBV challenge: To evaluate the protective efficacy of the developed DNA vaccines, all chickens were challenged with $100EID_{50}$ of the IBV SAIBk strain. Mortality, kidney infection, and percent protection of chickens after challenge were summarized in Table 1. No protection was obtained in the two control groups (pVAX1, PBS), where all chickens exhibited the typical symptoms of IBV infection and eventually died. In contrast, the majority of the chickens immunized with pVAX1-S1 (75%) or pVAX1-N (65%) were protected. Furthermore, protection (85%) in the pVAX1-S1/M/N group was higher than the other groups. RT-PCR of the collected kidney samples indicated that all chickens in the two control groups were positive for

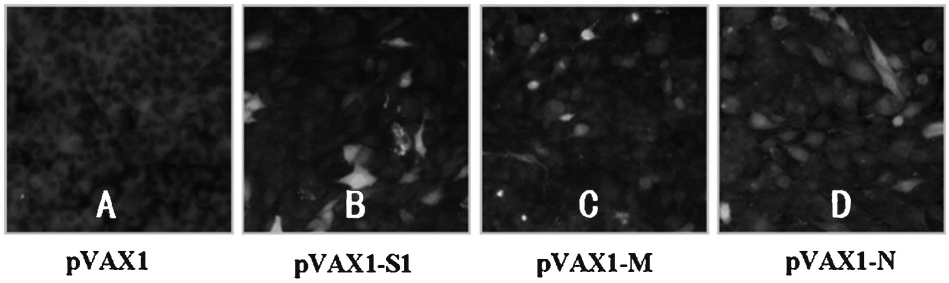

Fig. 2. Indirect-immunofluorescence detection of the expressed S1, M, and N proteins in Vero cells. (A) Cells transfected with the pVAX1 plasmid showed negative results. (B) Cells transfected with the pVAX-S1 plasmid showed positive results. (C) Cells transfected with the pVAX-M plasmid showed positive results. (D) Cells transfected with the pVAX-N plasmid showed positive results.

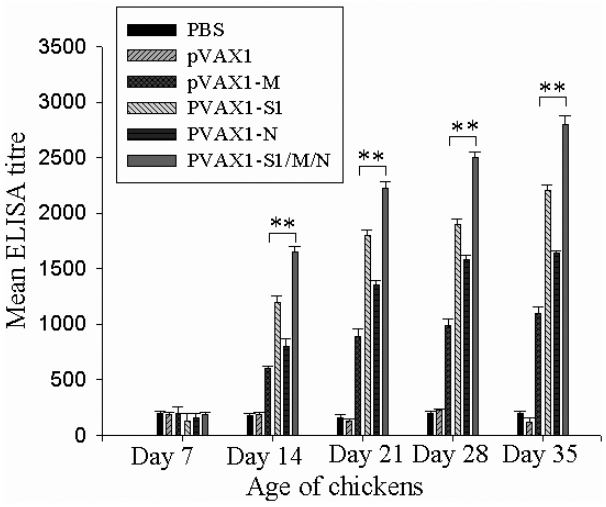

Fig. 3. Mean ELISA antibody titer of the different chicken groups vaccinated with different vaccines. ** P<0.01, significantly different ELISA titer at the given days compared to the other groups.

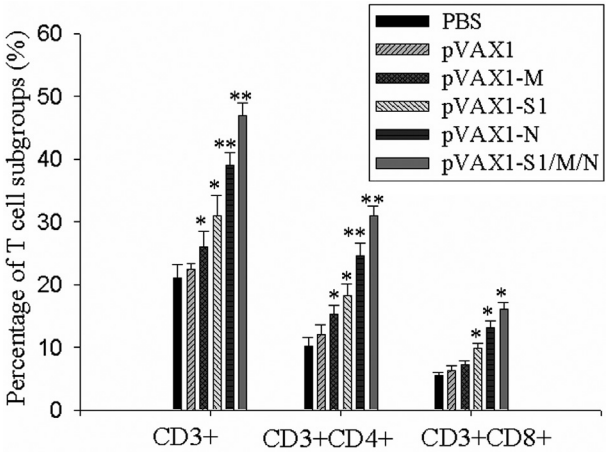

Fig. 4. Flow cytometry analysis of the percentage of $CD3^+$, $CD4^+CD3^+$, and $CD8^+CD3^+$ T-lymphocytes in the different vaccine inoculated groups. PBMCs were adjusted to 1×10^6 cells/100 μl. This test was performed at one week after boosting immunization. Statistical significance, * P<0.05, ** P<0.01.

IBV, while only 15% of chickens vaccinated with the combined DNA vaccine (pVAX1-S1/M/N) were positive, suggesting that the multivalent DNA vaccine offered a more reliable resistance against a virulent IBV challenge.

DISCUSSION

Immunization with DNA plasmids encoding for a determined antigen has been shown to be a promising new technology for generating immunity against viral, parasitic, and bacterial pathogens in a variety of animal species. In present study, we generated three expression plasmids targeting spike protein (S1), nucleocapsid protein (N), or membrane protein (M) for the development of potential DNA vaccine candidates against IBV. The S1, M, and N proteins of the IBV SAIBk strain were successfully detected in Vero cells transfected with pVAX1-S1, pVAX1-M, and pVAX1-N, respectively. Next, the immune response was evaluated in chickens immunized with the constructed DNA vaccines either alone or in a combined vaccine regimen. Each of the IBV target antigens in the vaccines played a protective role and elicited an immune response against IBV.

DNA vaccination with pVAX1-M alone elicited a small response in comparison with the response obtained from chickens immunized with pVAX1-S1 and pVAX1-N, as evaluated by ELISA, demonstrating that the M glycoprotein was the least immunogenic, and elicited the lowest titer of ELISA antibodies among the IBV constitutive proteins. In contrast, the chickens immunized with pVAX1-S1 showed the highest antibody response by ELISA in the three single gene DNA vaccine groups. It was demonstrated that the spike and not the membrane glycoprotein induced neutralizing antibodies and determined the serotype of the virus [13]. However, due to its amino acid sequence variation (20–25%), the S1 gene serotype usually differs, thus limiting its application as an IBV vaccine [2, 4].

To achieve a broader response against different IBV strains, cell mediated immunity (CMI) to IBV should be critical in IBV vaccine development, since it is considered a protective mechanism against IBV [7, 9]. In order to evaluate T-cell responses induced by recombinant plasmids, we analyzed peripheral blood lymphocytes using flow cytometry, and demonstrated that the percentage of $CD4^+$ and $CD8^+$

Table 1. The mortality and protection rate of different groups challenged by the virulent strain SIBK of IBV

Groups	No. deaths	No. IBV positive[a)]	Mortality[b)]	Protection rate[c)]
PBS	13/20	20/20	65%	0%
pVAX1	12/20	20/20	60%	0%
pVAX1-S1	2/20	5/20	10%	75%
pVAX1-M	6/20	12/20	30%	40%
pVAX1-N	3/20	7/20	15%	65%
pVAX1-S1/M/N	1/20	3/20	5%	85%

a) The number of chickens positive for IBV was determined by RT-PCR from the kidneys from dead and euthanized chickens. b) Mortality was recorded for each day after challenge and is presented as total number of dead chickens in each group. c) Percent protection was determined by the number of chickens negative for IBV divided by the total number of chickens.

T-lymphocyte subgroups in pVAX1-N vaccinated chickens were significantly higher than those in the pVAX1-S1 or pVAX1-M vaccinated chickens. The $CD8^+$ CTL have been reported to be critical in the control of infectious bronchitis in poultry [9, 27], while the $CD4^+$ T-cell responses may increase the proliferation, maturation, and functional activity of $CD8^+$ CTL, and directly produce the antiviral cytokines [17, 19]. Our results support an important role of N protein in protecting against IBV. Thus, the combined DNA vaccine with N protein may provide better protection against IBV. In the present study, the S1 and M proteins were also able to induce CMI responses. Although pVAX1-M alone elicited the lowest titers of antibodies by ELISA, its contribution to CMI responses supported a role in inhibition of virus replication.

To investigate the protection elicited by the DNA vaccines, chickens were challenged with a nephropathogenic strain of IBV. The chickens that received the combination of the three DNA vaccines showed a higher protection than the chickens immunized with the individual DNA vaccines, suggesting that a DNA vaccination with S1, M, and N genes in combination may provide a strong and complete protection against IBV. This observation is in line with the reports that the DNA vaccines in combination can elicit stronger humoral and cellular immune responses, thus resulting the more efficient protection against virus challenge [3, 15, 16, 23]. Present study is in contrast with a previous study that the combined DNA vaccines can reduce immunogenicity, which is referred to as "antigen competition", revealing that the detailed mechanism of multivalent DNA vaccine remained to be further investigated [26].

Taken together, our results directly demonstrate that multivalent DNA vaccine targeting S1, M, and N protein of IBV synergistically augmented the protective efficacy in chickens than individual plasmids. It is hoped that the results from the present study may help in providing further information on the potential usefulness of DNA vaccines against IBV.

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