Co-expression of IBV structural proteins and chicken interleukin-2 for DNA immunization

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ABSTRACT: The S1 gene of IBV and the chicken IL-2 gene were ligated together into a eukaryotic expression vector pCI-neo for co-expression evaluation *in vitro*. The recombinant plasmids were transfected into Vero cells, and the expression of targeted proteins was confirmed by an indirect immunofluorescent assay and immunocytochemistry assay. By repeating intramuscular injection several times, the co-expression plasmids provided chicks with some immune protection against viral infection.

Keywords: IBV; S1 gene; IL2; expression

Avian coronavirus infectious bronchitis virus (IBV) causes acute highly contagious disease in chicken flocks and is of great economic importance to the poultry industry (Schalk and Hawn, 1931; Bonnefoy et al., 1993; Cavanagh and Naqi, 1997). Generally, IBV causes respiratory, reproductive or renal symptoms. Traditional live vaccine and inactivated vaccine have been applied widely to prevent infectious bronchitis (IB) in chicken flocks. However, this has not prevented outbreaks of IB because of the variability and complicated serotype of IBV. A DNA vaccine is modifiable at the molecular level and is a potential candidate to substitute or supplement traditional vaccines. Towards this goal, It was necessary to evaluate how the structural proteins of IBV are expressed in eukaryotic cells and whether they could induce enough immune protection in animals.

IBV has four major structural proteins: Spike protein (S) which is composed of S1 and S2, membrane protein (M), nucleocapsid protein (N) and small envelope protein (E). S1 protein forms a spike outside the virion and is important for infection and for the high variation frequency of IBV (Cavanagh, 1983; Mockett et al., 1984; Cavanagh and Davis, 1986; Cavanagh et al., 1986; Koch et al., 1990). N protein combines with the chromosome inside the virion and is thought to play an important role in sustaining the structure and replication of the chromosome (Baric and Nelson, 1988; Collisson et al., 1995; Zhou et al., 1996; Denison et al., 1999). S1 protein and N protein might play important roles in inducing immune protection in vaccinated or infected chicks. For this reason it was reasonable to evaluate DNA immunization based on these genes.

Chicken interleukin-2 (IL-2), an important immune regulator, was confirmed to be able to enhance DNA immunization in some cases (Tang et al., 2008; Saade et al., 2008; Kumar et al., 2009). Evaluation of co-delivery of the IL-2 gene and IBV structural genes might help us in optimizing a potential DNA vaccine for IBV.

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MATERIAL AND METHODS

Gene cloning and recombinant plasmid construction

Primers as described below were designed to clone the S1 gene, N gene and IL-2 gene. RT-PCR was performed as described previously (Zhang et al., 2002; Zhou et al., 2004, 2005):

pS1up: 5'-GCGAATTCATGGCTTTGTATGACAGTAGTTCT-3'

pS1down: 5'-CGTCTAGATTAATAACTAACATAAGGGCA-3'

pNup: 5'-CGGAATTCATGGCAAGCGGTAAAGCAG-3'

pNdown: 5'-CCTCTAGATTACTCAAAGTTCATTCTCTCC-3'

pILup: 5'-GGAATTCGCATCTCTATCATCAGCAAAA-3'

pILdown: 5'-TTTCTAGATTATTTTTGCAGATATCTCAC-3'

The S1 gene, N gene, and IL2 gene were ligated with mammalian expression vectors pCI-neo (Promega, USA) respectively at *EcoR* I/*Xba* I sites. The recombinant plasmids were named pCI-neo-S1, pCI-neo-N and pCI-neo-IL2 respectively (Figure 1).

The co-expression plasmids pCI-neo-IL2-S1 containing chicken IL2 gene and IBV S1 gene were then constructed. In pCI-neo-IL2-S1, each gene was provided with a CMV promoter and a SV40 Poly A terminator for non-fusion expression (Figure 2). Briefly, a linear DNA segment composed of "IL2 + Poly A signal" was prepared by PCR using the above pCI-neo-IL2 as a template. Another linear DNA segment composed of "Promoter + S1" was prepared by PCR using pCI-neo-S1 as a template. The two above segments were then ligated with pCI-neo which had been digested with the enzyme *Mlu* I.

The recombinant plasmids were all identified by PCR, enzyme digestion and sequencing.

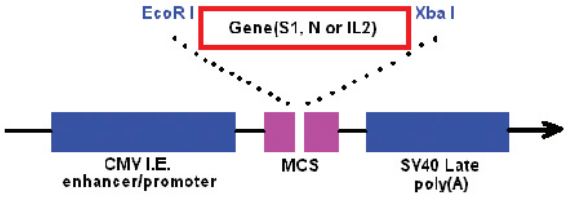

Figure1. Schematic structure of recombinant plasmid pCI-neo-S1, pCI-neo-N, and pCI-neo-IL2. The cDNA sequence of the S1 gene, N gene and IL2 gene were inserted into the MCS of the vector pCI-neo between *EcoR* I and *Xba* I sites

Super-pure plasmid preparation

Plasmids of super purity were prepared with a super purification plasmid extraction kit according to the instruction manual. Briefly, buffer E1 containing bacteria was mixed with buffer E2. After 5 min of incubation at room temperature, the suspension was mixed with buffer E3 for 10 min and centrifuged at 12 000g. The supernatant was transferred onto a column for unprompted flowage. After washing the column twice, elution buffer was added to elute the target DNA.

Transfection of Vero cells

The super pure plasmids prepared above were transfected into Vero cells cultured in 24-well plates using the Lipofectamine 2000 transfection kit. Briefly, Vero cells were cultured in 24-well plates up until about 90% coverage. 50 µl of MEM media containing 0.8 µg of DNA was then mixed with an equal volume of MEM containing 2 µl Lipofectamine 2000 liquid for 20 min of incubation at room temperature. The prepared mixture was added into 24-well plates drop by drop. Cells transfected with DNA were cultured for an additional 48 hours.

Detection of the recombinant proteins

An indirect fluorescence assay (IFA) was performed 24 h after transfection to detect the expressed proteins on 24-well plates. Briefly, after washing three times with PBS, the cells were added to a mixture of acetone and methanol for 45 min of immobilization at 20°C. The cells were then incubated for 90 min with 5% milk powder solution at 37°C and were washed 3 times. Primary antibody (raised by the Institute of Preventive Veterinary Medicine, Zhejiang University, China) was added for 90 min of incubation at 37°C. After washing, the secondary

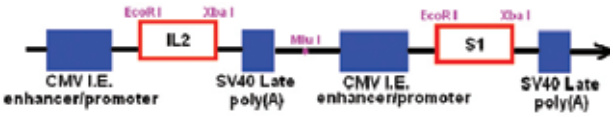

Figure 2. Schematic structure of the recombinant co-expression plasmid pCI-neo-IL2-S1. The chicken IL2 gene and IBV S1 gene were constructed in a single plasmid. Each of the genes sits between a CMV enhancer/promoter and a SV40 poly(A) signal sequence

antibody labeled with FITC was added for 90 min of incubation and the results were observed under an inverted fluorescence microscope.

As a further check, a immunocytochemistry assay (ICC) was also performed The protocol for the ICC was similar to the IFA above except that the secondary antibody was labeled with HRP, and finally the results were detected by color development of AEC reagentIPMA.

Chick protection experiment

Three-week-old chicks were divided into seven groups randomly, with ten in each group. As shown in Table 1, chicks in Group 1 were intramuscularly injected with pCI-neo as a negative control. Chicks in Group 2 to Group 6 were vaccinated intramuscularly with 200 μg of pCI-neo-S1, pCI-neo-N, pCI-neo-IL2-S1, PCI-neo-S1 + pCI-IL2, PCI-neo-S1, and pCI-neo-IL2 + pCI-neo-N, respectively. All chicks were vaccinated three times at weekly intervals. Chicks in Group 7 were either intranasally or ocularly administered a commercial live IBV vaccine (strain H120) in accordance with the manufacturer's instruction (Hangzhou Jianliang Veterinary Bioproducts Co. Ltd., China).

All of the vaccinated chicks were intranasally challenged with 50 μl of virulent IBV (strain M41; $10^{4.0}$EID50/0.1 ml; Chinese Institute of Veterinary Drug Control) at 14 days after the third immunization and were observed daily for 10 days. After challenge with the virulent IBV, the clinical signs of chickens were recorded according to the following criteria described by Collisson et al. (2000):

– = no clinical signs of illness

+ = sneezing or nasal discharge

++ = plus dyspnea or rales

+++ = plus lack of appetite, pale color, or ataxta

++++ = death

The recording terminated 10 days after challenge.

Ten days after challenge, birds were all euthanased and necropsied to reveal the pathological changes caused by IBV infection. Meanwhile, an attempted re-isolation of the virus from trachea, lung or kidney was performed. The re-isolation of IBV (strain M41) was confirmed via amplification of the S1 gene by RT-PCR. (Zhou et al., 2004).

RESULTS

Construction of recombinant plasmids

PCR using the recombinant plasmids as templates and *EcoR* I/*Xba* I endonuclease digestion experiment confirmed that pCI-neo-S1, pCI-neo-N and pcDNA3-S1 were successfully constructed. In both PCR and enzyme digestion experiments, the S1 gene, 1.65 kb in length or the N gene of 1.23 kb in length were confirmed (Figure 3).

Expression of S1 gene and N gene in Vero cells

IFA confirmed that the S1 gene and N gene were expressed in Vero cells transfected with pCI-neo-S1, pCI-neo-N or pCDNA3-S1. The positive cells showed green fluorescence while the surrounding negative cells displayed none. Interestingly, it was observed that the N protein seemed to be prone to

Table 1. Immunization procedure and protection rate of the challenge experiments

Group	Vaccination method	Vaccination procedure					Clinical protection	Necropsy protection
		14D	21D	28D	42D	52D		
1	pCI-neo (200 mg)	first vaccination	second vaccination	third vaccination	challenge	necropsy	++(2); +++(8)	1/10
2	pCI-neo-S1 (200 mg)						–(6); ++(2); +++(2)	6/10
3	pCI-neo-N (200 mg)						–(5); ++(2); +++(3)	5/10
4	pCI-IL2-S1 (200 mg)						–(7); +++(3)	6/10
5	pCI-S1 (200 mg) + pCI-IL2 (200 mg)						–(7); ++(2); +++(1)	7/10
6	pCI-S1 (200 mg) + pCI-IL2 (200 mg) + pCI-N (200 mg)						–(7); ++(1); +++(2)	7/10
7	live valine (50 ml)						–(9); ++(1)	9/10

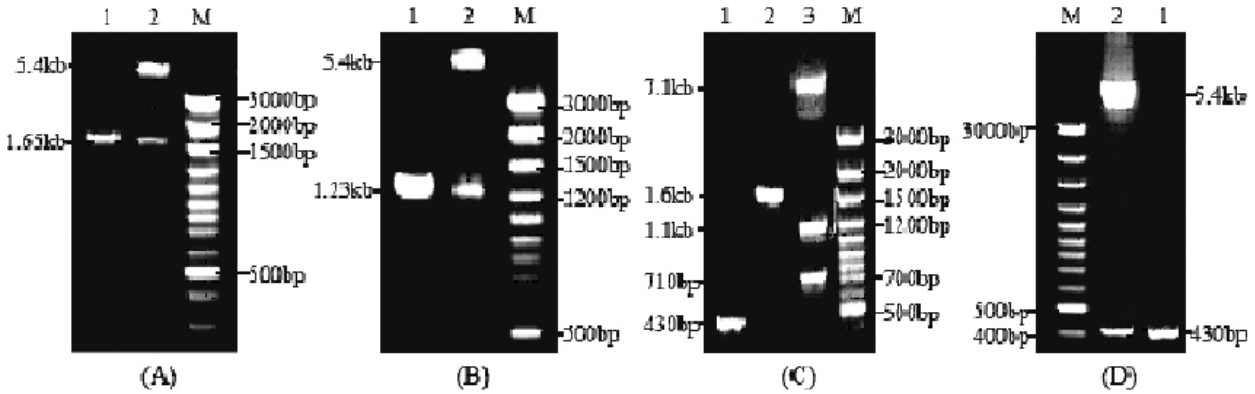

Figure 3. Identification of recombinant plasmids. (**A**) pCI-neo-S1 identification: 1 = PCR product, 2 = EcoR I/Xba I digested product; (**B**) pCI-neo-N identification: 1 = PCR product, 2 = EcoR I/Xba I digested product; (**C**) pCI-neo-IL2-S1 identification: 1 = PCR for IL2, 2 = PCR for S1, 3 = EcoR I/Mlu I digested product; (**D**) pCI-neo-IL2: 1 = PCR product, 2 = EcoR I/Xba I digested product

nuclear localisation, as the nucleus of N protein-expressing cells always showed more intense fluorescence than the cytoplasm. The S1 protein was observed only in the cytoplasm (Figure 4).

ICC also confirmed the results observed in IFA. The S1 and N genes were successfully expressed in some of the transfected cells which showed red color. The control cells which didn't express the foreign proteins showed light yellow color (Figure 5).

Chick protection experiment

As shown in Table 1, intramuscular administration of plasmids containing the S1 gene or N gene successfully provided chicks with some immune protection. An approximately 50% observable clinical protection was recorded for pCI-neo-N, about 60% observable clinical protection was recorded for pCI-neo-S1, and about 70% observable clinical protection was recorded when IL2 was administrated together

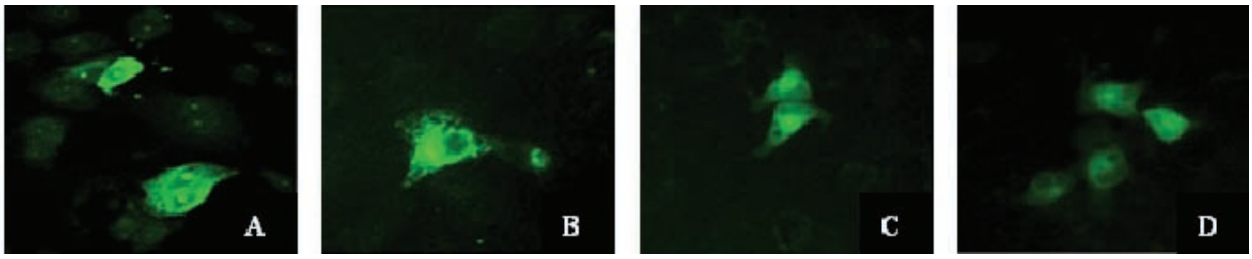

Figure 4. Detection of the expression of recombinant plasmids in Vero cells by IFA (200×). A = pCI-neo-N; B = pCI-neo-S1; C = pCI-neo-IL2; D = pCI-neo-IL2-S1

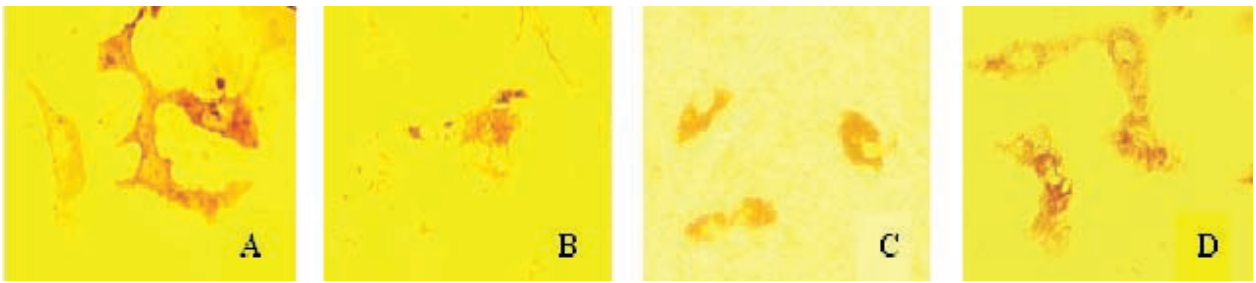

Figure 5. Detection of the expression of recombinant plasmids in Vero cells by ICC (200×). A = pCI-neo-N; B = pCI-neo-S1; C = pCI-neo-IL2; D = pCI-neo-IL2-S1

with the S1 gene. However, the protection ratio was a bit lower at necropsy level. Co-delivery of different plasmids seemed to induce better immune protection. It was also noticed that the traditional live vaccine induced better immune protection compared with the DNA vaccine.

DISCUSSION

DNA vaccines having been a focus of research for many years, but few are yet applied. For IBV, some efforts have also been made to evaluate the feasibility of gene immunization using its structural genes (Kapczynski et al., 2003; Tang et al., 2008). Challenges to developing a DNA vaccine against IBV to substitute traditional vaccines include their stability, protection ratio and economic costs. Kapczynski et al's study indicated that the S1 gene might induce some resistance in chicks against virus challenge despite the fact that no neutralizing antibody or ELISA antibody was detected (Kapczynski et al., 2003). Results such as these were disappointing since a high antibody titer is to be expected of most vaccines. It is therefore necessary to optimize such factors as targeted genes, immunization routes, vectors, adjuvant, etc.

This study confirmed that the S1 gene and N genes of IBV carried on the pCI-neo vector can be expressed in eukaryotic host cells. The expression was observed from 24 h through 48 h after transfection. The S1 gene and N gene showed different *in vitro* expression characteristics in this study. In IFA and ICC, the two genes showed different subcellular positioning characteristics. All of the cells expressing N proteins showed abundant expression inside the nucleus, while the S1 protein was only found to be located in the cytoplasm. The results indicate that the N protein might possess a certain nuclear targeting sequence. In addition, More N proteins than S1 proteins were detected in transfected Vero cells when an equal quantity of plasmids and same operation methods were used. In another study, it was also noticed that the expression of the S1 protein in *E. coli* was difficult to detect because of its low levels (unpublished) while the N protein showed high expression levels (Zhang et al., 2005). Butit was necessary to try to improve its expression level. The S1 gene was generally thought to be the optimal candidate for a DNA vaccine because it contained important neutralizing epitopes.

The S1 gene was generally thought to be more important than the N gene for immunization. But in the chicks protection experiments, both the S1 gene and N gene provided relative high immune protection according to the clinical records. A relatively high expression level might be one of the factors which enhanced the immunogenicity of the N protein. Cellular immunization is thought to play an important role for DNA vaccines, so it can be presumed that genes that possess less neutralizing epitopes might also induce enough immune reaction through high levels of expression (Kapczynski et al., 2003). Moreover, it has been noticed that a high purity and proper carrier for plasmids is important for DNA immunization because of it effects on transfection ratios. Unfortunately, the protection effect of the DNA vaccine in this study was somewhat poorer than a traditional live vaccine even after three intramuscular injections with high purity plasmids.

As a simple and economic administration method for an IBV DNA vaccine, co-delivery of the S1 gene and IL-2 gene in one plasmid was confirmed to be effective in this study. Although it was found that the protection ratio of DNA immunization was lower and the costs were much higher, it still provided a potential substitute for traditional live vaccines because a DNA vaccine can be designed and modified easily in a laboratory and is a suitable solution to the high variance of IBV.

REFERENCES

Baric R.S., Nelson G.W. (1988): Interactions between coronavirus nucleocapsid protein and viral RNAs: Implications for viral transcription. Journal of Virology, 62, 4280–4287.

Bonnefoy L., Bouquet J.F., Picault J.P., Chappuis G. (1993): Characterization of IBV variant strain PL 84084 isolated in France. Advances in Experimental Medicine and Biology, 342, 395–397.

Cavanagh D. (1983): Coronavirus IBV: further evidence that the surface projections are associated with two glycopolypeptides. Journal of General Virology, 64, 2577–2583.

Cavanagh D., Davis P.J. (1986): Coronavirus IBV: removal of spike glycopolypeptide S1 by urea abolishes infectivity and haemagglutination but not attachment to cells. Journal of General Virology, 67, 1443–1448.

Cavanagh D., Naqi S.A. (1997): Infectious bronchitis. In: Calnek B.W., Barnes H.J., Beard C.W., McDougald L R.,

Saif Y.M. (eds.): Diseases of Poultry. Iowa State University Press, Iowa, USA. 511–526.

Cavanagh D., Davis P.J., Darbyshire J.H., Peters R.W. (1986): 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. Journal of General Virology, 67, 1435–1442.

Collisson E.W., Williams A.K., Chung S.I., Zhou M. (1995): Interactions between the IBV nucleocapsid protein and RNA sequences specific for the 3' end of the genome. Advances in Experimental Medicine and Biology, 380, 523–528.

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. Developmental & Comparative Immunology, 24, 187–200.

Denison M.R., Spaan W.J., van der Meer Y., Gibson C.A., Sims A.C., Prentice E. (1999): The putative helicase of the coronavirus mouse hepatitis virus is processed from the replicase gene polyprotein and localizes in complexes that are active in viral RNA synthesis. Journal of Virology, 73, 6862–6871.

Kapczynski D.R., Hilt D.A., Shapiro D., Sellers H.S., Jackwood M.W. (2003): Protection of chickens from infectious bronchitis by in ovo and intramuscular vaccination with a DNA vaccine expressing the S1 glycoprotein. Avian Disease, 47, 272–285.

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. Journal of General Virology, 71, 1929–1935.

Kumar S., Ahi Y.S., Salunkhe S.S., Koul M., Tiwari A.K., Gupta P.K., Rai A. (2009): Effective protection by high efficiency bicistronic DNA vaccine against infectious bursal disease virus expressing VP2 protein and chicken IL-2. Vaccine, 27, 864–869.

Mockett A., Cavanagh D., Brown T. (1984): Monoclonal antibodies to the S1 spike and membrane proteins of avian infectious bronchitis coronavirus strain Massachusetts M41. Journal of General Virology, 65, 2281–2286.

Saade F., Buronfosse T., Pradat P., Abdul F., Cova L. (2008): Enhancement of neutralizing humoral response of DNA vaccine against duck hepatitis B virus envelope protein by co-delivery of cytokine genes. Vaccine, 26, 5159–5164.

Schalk A.F. Hawn M.C. (1931): An apparently new respiratory disease of baby chicks. Journal of the American Veterinary Medical Association, 78, 413–422.

Tang M., Wang H., Zhou S., Tian G. (2008): Enhancement of the immunogenicity of an infectious bronchitis virus DNA vaccine by a bicistronic plasmid encoding nucleocapsid protein and interleukin-2. Journal of Virology Methods, 149, 42–48.

Zhang D.Y., Zhou J.Y., Chen J.G., Ding H.M., Shen X.Y. (2002): Cloning and sequence analysis of nucleocapsid gene of x-strain of nephri pathogenic infectious bronchitis virus (in Chinese). Chinese Journal of Veterinary Science, 22, 529–532.

Zhang D.Y., Zhou J.Y., Fang J., Hu J.Q., Wu J.X., Mu A.X. (2005): An ELISA for antibodies to infectious bronchitis virus based on nucleocapsid protein produced in *Escherichia coli*. Veterinarni Medicina, 50, 336–344.

Zhou M., Williams A.K., Chung S.I., Wang L., Collisson E.W. (1996): The infectious bronchitis virus nucleocapsid protein binds RNA sequences in the 3' terminus of the genome. Virology, 217, 191–199.

Zhou J.Y., Zhang D.Y., Ye J.X., Cheng L.Q. (2004): Characterization of an avian infectious bronchitis virus isolated in China from chickens with nephritis. Journal of Veterinary Medicine, Series B, 51, 147–152.

Zhou J.Y., Chen J.G., Wang J.Y., Wu J.X., Gong H. (2005): cDNA cloning and functional analysis of goose interleukin-2. Cytokine, 30, 328–338.

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