An ELISA for antibodies to infectious bronchitis virus based on nucleocapsid protein produced in *Escherichia coli*

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ABSTRACT: The nucleocapsid (N) gene of infectious bronchitis virus (IBV) strain X isolated in China was expressed in *E. coli* and was purified as a recombinant protein. An indirect ELISA assay (*N*-ELISA) for antibody detection was established using the purified recombinant nucleocapsid protein. Antigen coating conditions and serum dilution for the *N*-ELISA were optimized. The S/P ratio of the absorbency value was calculated in the *N*-ELISA to evaluate the antibody level of chicken serum. In an experiment to test field samples for antibody detection, the *N*-ELISA assay shared 95.7% identity of total positive ratio with the commercial ELISA kit. It indicated that the *N*-ELISA assay, which was safer and easier to prepare than traditional methods, was a good candidate for evaluation of IB vaccine efficiency and virus exposure.

Keywords: infectious bronchitis virus; nucleocapsid protein; *in vitro* expression; ELISA

Infectious bronchitis (IB) is an acute and highly contagious disease which occurs worldwide and results in severe economic loss in the poultry industry (Schalk and Hawn, 1931; Collisson et al., 1992). Attenuated live vaccines are used widely to control IB, but the occurrence of IB in vaccinated flocks is continuously reported because the avian coronavirus infectious bronchitis virus (IBV) varies frequently. IBV exists as many serotypes and the variant strains even showed different tissue tropism (Albassam et al., 1986; Parsons et al., 1992; Fulton et al., 1993; Li and Yang, 2001; Zhou et al., 2003b, 2004b).

The ELISA assay is a convenient method for monitoring of both the immune status and virus infection in chicken flocks. Several commercial ELISA kits for IBV specific antibodies detection are already available, which used inactivated virions as coating antigen. However, the propagation and purification of IBV is tedious, unsafe and expensive. Unlike full virions, recombinant proteins produced via gene engineering detect only specific antibodies to one certain protein and are safer, easier to produce and less expensive. The genome of IBV encodes three major structural proteins: the spike protein (S), the membrane (M) and the nucleocapsid protein (N). The S protein carries serotype-specific sequences and neutralization epitopes and is involved with infectivity. The N protein is bound with the genome of IBV located inside the virions and plays an important role in viral replication and assembly (Zhou and Collisson, 2000). The N protein is produced early and abundantly during virus infection (the molar ratio of N : S is about 6 : 1) (Cavanagh, 1983). In addition, the N protein is highly conserved among different IBV strains

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(Williams et al., 1992). Therefore, the N protein was thought to be an appropriate diagnostic reagent for antibody detection. To solve the safety problem of the traditional vaccines, researchers are seeking a new generation of vaccines such as subunit and DNA vaccines (Seo et al., 1997; Kapczynski et al., 2003). ELISA assays based on recombinant protein would also be useful in such studies because they could differentiate antibodies against DNA or subunit vaccines from those caused by whole virion infection.

Currently, nephropathogenic variant strains of IBV are thought to be of great importance in China where they can cause high mortality even in vaccinated young chicken flocks. Nucleotide sequence showed that these variant strains shared low identity with traditional strains of IBV (Zhou et al., 2003b, 2004b). It is urgent to understand more about these strains and to develop diagnostic methods which suit them better. In the present study, the N gene of a nephropathogenic strain X of IBV isolated in China was expressed in *E. coli.* and an ELISA assay based on the recombinant N protein was evaluated.

MATERIAL AND METHODS

Source of viruses and antibodies. IBV isolate ZJ971 (GenBank No. AF352308, AF352311) isolated from chicken proventriculus and the nephropathogenic IBV isolate X (GenBank No. AY043221, AY043315) were stored in our laboratory (Zhou et al., 2003a, 2004a). Antisera against IBV strains X, ZJ971, H52 and H120, respectively were prepared in our laboratory (Zhou et al., unpublished data). Antisera against avian influenza virus (H9N2, AIV), infectious bursal disease virus (IBDV), Newcastle disease virus (NDV) and IBV strain M41 were provided by Ha'erbin Veterinary Research Institute, Chinese Academy of Agricultural Science (Ha'erbin, Heilongjiang province, China). Field serum samples were collected from three poultry farms in Zhejiang, China where the chickens had been vaccinated with commercial live attenuated IB vaccines (H52 and H120). Chickens in Farms 1, 2 and 3 were 140-day-old, 110-day-old and 120-day-old, respectively.

Expression and purification of N protein of IBV. The open reading frame (ORF) consisting of 1 230 nucleotides was subcloned by PCR using the upstream primer (pIBVN1) 5'-CGGAATTCATG GCAAGCGGTAAAGCAG-3' containing the *Eco*R I site and the downstream primer (pIBVN2) 5'-CCAAGCTTACTCAAAGTTCATTCTCTCC-3' containing the *Hind* III site, from the recombinant vector pBS-N containing the N gene of IBV isolate X (Zhang et al., 2002). PCR was performed by 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and polymerization at 72°C for 2 minutes. The final polymerization was conducted at 72°C for 10 minutes. ORF of the N gene was ligated into the vector pBAD/his B (Invitrogen Co., CA, USA) between *EcoR* I and *Hind* III sites (Figure 1). The resultant expression vector pBAD/his B-N was then transformed into *E. coli* strain LM194 (Invitrogen) and was identified by PCR, restriction enzyme digestion and sequencing.

The recombinant *E. coli* strain LM194 was cultured in RM medium (2% casamino acids, 1 mM $MgCl_2$, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl, 0.2% glucose) and was then induced with 0.02% final concentration of L-arabinose to express the N protein according to the manufacture's protocol (Invitrogen). One LMG194 clone containing only the parent vector pBAD/his B was used as negative control. Expression of N protein was checked by SDS-PAGE and western blot assays

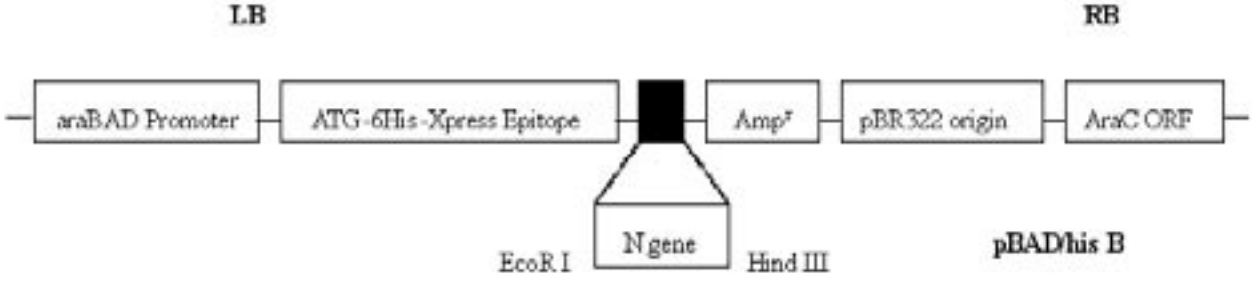

Figure 1. Schematic structure of recombinant plasmid pBAD/his B-N. The cDNA sequence of N gene was inserted into the MCS of the vector pBAD/his B between the *EcoR* I and *Hind* III sites. Xpress epitope and 6His were added to the N terminus of N gene

(Sambrook and Russell, 2001). Briefly, bacterial pellets collected by centrifugation were resuspended in 100 µl 1× loading buffer and boiled for 10 minutes. After centrifugation, 10 µl supernatant was taken and analyzed by 12% SDS-PAGE gel. The gel was transferred to a nitrocellulose (NC) membrane using Hoefer SemiPhor semi-dry transfer unit for 1.5 hrs according to the operation manual (Amersham Pharmacia Biotech Inc., NJ, USA). The NC membrane was blocked with 5% skim milk (SM) and incubated respectively at 37°C for 1 hour with anti-X press monoclonal antibody (Invitrogen) or antisera against IBV strains X, M41, ZJ971, H52 and H120. After washed three times with TBST buffer, the NC membrane was incubated with HRP-labeled goat anti-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Maryland, USA) or HRP-labeled rabbit anti-chicken IgG (Dingguo Biotech Inc., Beijing, China). Finally, the color reaction is developed with the substrate TMB (Promega, Madison, WI, USA).

Purification of the N protein was performed with a Nickel affinity column according to the operation manual (Qiagen, Germany). Briefly, the bacteria samples were suspended in 8M urea at pH 8.0 for ultrasonication. After centrifugation at 1 000 × g, the supernatant was collected and combined with Nickel column material for 1 hour. Then the proteins combined with the Nickel column were washed with 8 M urea at various pH values (pH 6.3, pH 5.9, and pH 4.5) in turn. All of the proteins eluted were collected separately and checked by SDS-PAGE. Finally, the bradford method was used to determine the concentration of the purified N protein (Walker, 1996).

***N*-ELISA procedure**. Chicken antisera to IBV strain M41 and SPF chicken negative sera were used to optimize the *N*-ELISA procedure. Ninety six-well ELISA plate (Canada JET Biochemicals Int'I. Inc., Toronto, Canada) was coated with 100 µl appropriately diluted recombinant N protein ranging from 15.2 µg/ml to 0.48 µg/ml and incubated at 37°C for 2 hours. Washed 3 times with TBST buffer, the plate was blocked with 5% skim milk, antisera diluted with 0.1% bovine serum albumin (BSA) were added and the plate was incubated at 37°C for 1 hour. After washing 3 times with TBST buffer, 100 µl HRP-labeled rabbit anti-chicken IgG was added to the plate and incubated at 37°C for 1 hour. After washing 4 times, 50 µl TMB was added to the plate followed by exposure for 10 minutes. The reaction was terminated with 2M H_2SO_4 and the OD_{450} value was then read with Elx800 Universal Microplate Reader (Bio-Tek Instruments, Inc., Winooski, VT, USA). Every sample was repeated in 3 wells and the mean value was calculated. S/P ratio was used as a standard for judgement. S/P = (OD_{450} of sample – OD_{450} of negative control)/(OD_{450} of positive kontrol – OD_{450} of negative control). S/P ≥ 0.2 was judged into positive and S/P ≤ 0.2 was judged into negative.

Stability and specificity of the *N*-ELISA procedure. To evaluate the stability of the *N*-ELISA procedure, three batches of N protein prepared separately were used as coating antigen to detect a positive antiserum against IBV strain M41. In each experiment, six wells were repeated and the mean value was calculated for evaluation. To evaluate the specificity of the *N*-ELISA procedure, antisera against IBV strains M41, H52, H120, X, ZJ971 and antisera against AIV, IBDV, NDV and *E. coli*, respectively were tested with the *N*-ELISA assay.

Field sample detection. Ninety five serum samples collected from three vaccinated chicken flocks in separate poultry farms (Farm 1, Farm 2 and Farm 3) in Zhejiang, China were examined with both the *N*-ELISA assay and a commercial ELISA kit (IDEXX, Westbrook, Maine, USA). The positive ratio and antibody titer of sera in each farm were compared.

Bioinformatical softwares and statistical analysis. Bioinformatical software OMIGA 2.0 (Oxford Molecular Ltd., USA) was used to analyze the gene sequence and to predict the molecular weight of protein. Statistical analysis was performed with ANOVA method (SAS 1996). Difference was considered to be significant when $P < 0.05$.

RESULTS

Expression and purification of N protein

In the *in vitro* expression test for the N protein, all of the bacteria samples were collected from 2 to 4 hours after IPTG induction. Subsequently, the bacterial lysates and purified proteins prepared with nickel affinity column chromatography system were tested. A fusion protein with a molecular weight of about 50 kd was presented in SDS-PAGE and western blotting (Figure 2). In addition, a band of approximately 46 kd fusion protein and several smaller molecular weights of fusion protein bands were also noticed. The smaller molecular weight

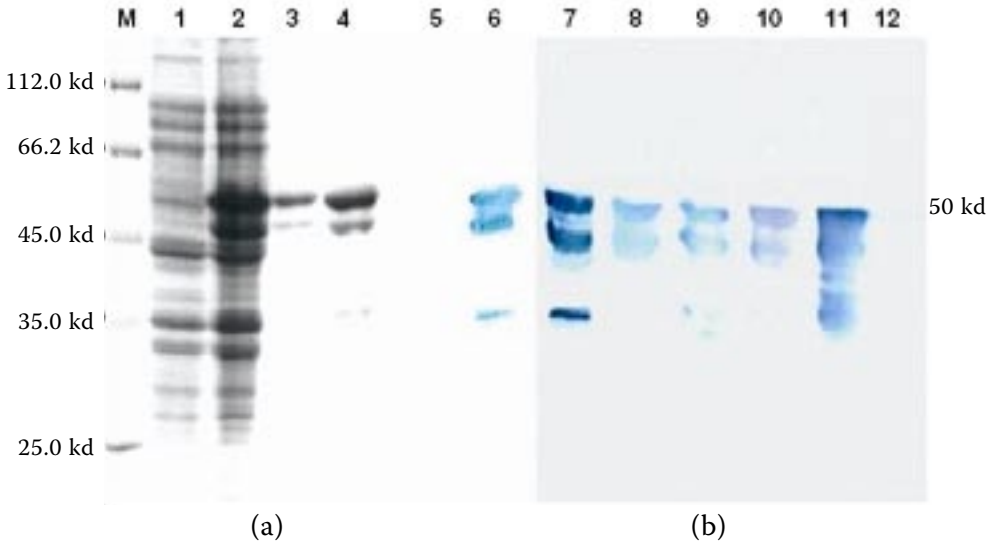

Figure 2. Expression and purification of N protein. (**a**) SDS-PAGE Identification of the N protein. Lane M – molecular weight markers; lane 1 – negative control *E. coli* LMG194 strain which was transformed with pBAD/his B vector; lane 2 – recombinant *E. coli* LMG194 strain presented the N protein with a molecular weight of about 50 kd; lane 3 and lane 4 – the purified N protein. (**b**) Western blotting identification of the N protein, epitope or polyvalent antisera against IBVs: Lane 5 – the mAb against X press did not react with negative control *E. coli* LMG194 strain; lane 6 – the N protein reacted with mAb to X press; lane 7 to lane 11 – the N protein recognised respectively by antisera against IBV strains M41, H52, H120, ZJ971 and X; lane 12 – SPF chicken negative serum did not react with recombinant *E. coli* LMG194 strain

protein could also react with the monoclonal antibody against X press epitope or antisera against IBV strains X, M41, H52, H120, and ZJ971 (Figure 2), indicating that they were all degraded segments of the original N protein. To make sure whether decreasing culture temperature after IPTG induction could prevent the presumed degradation of N protein, attempts were made to culture the bacteria at 25°C and 30°C instead of 37°C for 4–8 hours. However, SDS-PAGE and western blot analysises presented similar results as those in Figure 2.

The most efficient N protein expression was observed four hours after induction and the proportion of the expressed N protein in total bacterial proteins was about 20%. This indicated that the N protein of IBV was readily expressed in the pBAD/his B system. Both denatured and native methods were used to try to purify the N protein with a nickel column as described in the manufacture's protocol. Similar purity of the N protein was observed in both methods, but the yields of the eluted soluble N protein were about 18.24 mg/l bacterial culture in the denatured method and 12.37 mg/l bacterial culture in the native method respectively, as accessed by the Bradford method.

Optimization of *N*-ELISA procedure

Recombinant N protein was immobilized onto ELISA plates in serial two-fold dilutions from 15.2 μg/ml to 0.48 μg/ml and chicken antisera against IBV strain M41 or negative control chicken serum was also diluted in serial two-fold dilutions from 1 : 50 to 1 : 400 for optimization. It was found that the highest P/N value was obtained when the N protein was diluted to 1.9 μg/ml, and chicken anit-IBV positive (strain M41) and negative sera were diluted to 1/100, respectively (Figure 3).

To determine the optimal conjugate dilution in the *N*-ELISA, after the concentration of the N protein, chicken anti-IBV positive and negative sera were fixed onto the ELISA plate, the different concentrations of HRP-labeled rabbit anti-chicken IgG were added onto the ELISA plate at the dilutions of 1 : 400, 1 : 600, 1 : 800, 1 : 1 000 and 1 : 1 200. Data shown in Figure 4a indicates that 1 : 800 was the most appropriate dilution of the HRP-labeled rabbit anti-chicken IgG.

To test the optimal coating buffer, NaOH (pH13), 0.05M Bicarbonate/Carbonate buffer(pH 9.6), 0.1M Tris-HCl (pH 8.5), PBS (pH 7.4) and HCl (pH 4.6)

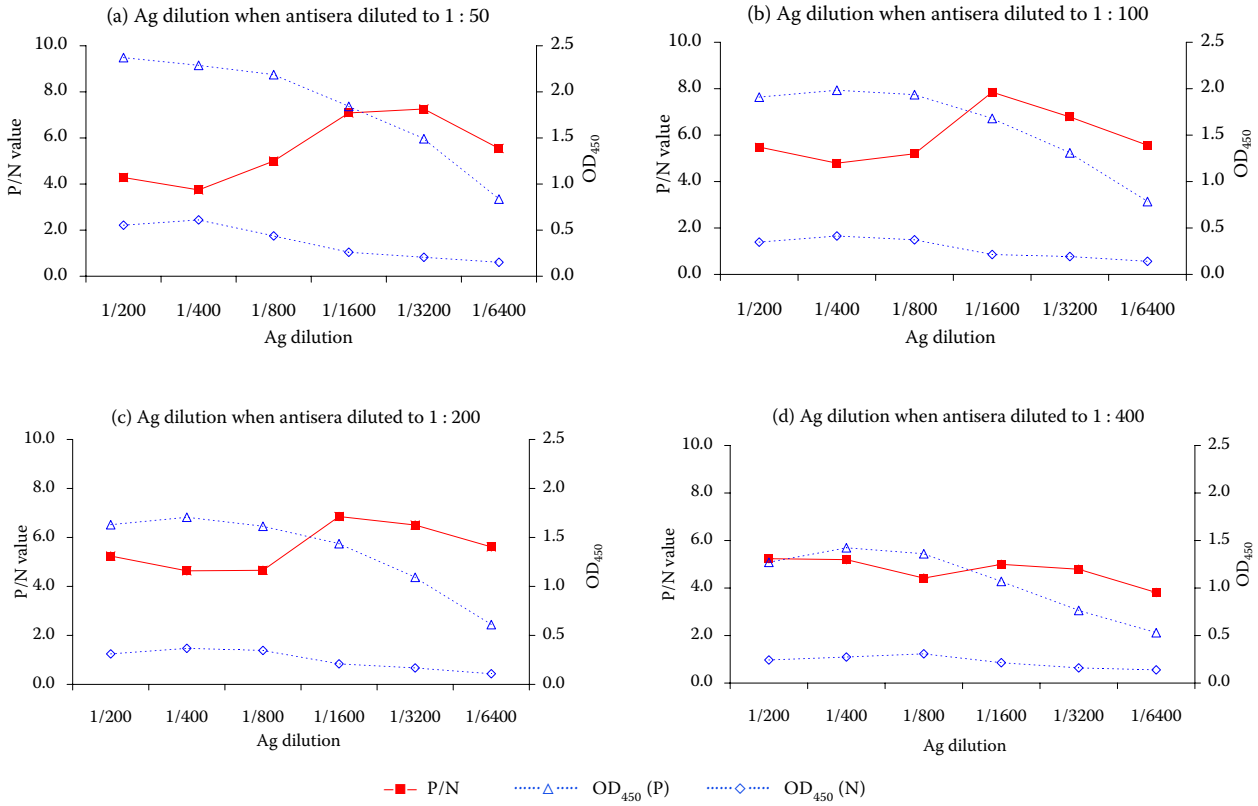

Figure 3. Optimization of the N protein and sera dilutions. The N protein was diluted from 15.2 μg/ml to 0.48 μg/ml while antisera were diluted to 1 : 50, 1 : 100, 1 : 200 and 1 : 400. The optimal dilutions of the N protein and sera were respectively 1.9 μg/ml and 1/100

was screened, it was shown that 0.1M Tris-HCl (pH 8.5) was the best coating buffer for immobilization of the N protein (Figure 4b). In the experiment of blocking buffer selection, six candidate blocking buffers (1% BSA, 0.5% BSA, 0.25% BSA, 10% skim milk, 5% skim milk and 2.5% skim milk) were compared, it was found that 1% BSA was the optimal blocking buffer for the *N*-ELISA (Figure 4c). Finally, after the ELISA plates was exposed for from 5 to 20 minutes in TMB solution, the result shown in Figure 4d indicated that the appropriate time of color development is 10 minutes with TMB solution in the *N*-ELISA procedure.

Specificity and stability of the *N*-ELISA assay

The S/P ratio ($\bar{x}$ ± SD) for IBV strains M41, X, ZJ971, H52, H120 was respectively 0.690 ± 0.023, 1.069 ± 0.021, 0.787 ± 0.024, 0.718 ± 0.026, and 0.646 ± 0.034 whereas the S/P ratio ($\bar{x}$ ± SD) for AIV(H9), IBDV, NDV, and *E. coli* (O78) was respectively 0.175 ± 0.011, 0.150 ± 0.026, 0.161 ± 0.019, and 0.148 ± 0.027. The results above indicated that the *N*-ELISA assay was specific and appropriate for detection of antibodies against the different IBV strains.

To determine the stability of the *N*-ELISA assay, three batches of recombinant IBV-N protein prepared separately were used as coating antigen and antiserum against IBV strain M41 was used a reference positive serum, the result showed that the S/P ratio ($\bar{x}$ ± SD) was 0.862 ± 0.008, 0.867 ± 0.046 and 0.866 ± 0.054 respectively, indicating that the difference between the three groups was not significant ($P > 0.05$) and the *N*-ELISA assay was stable.

Field samples detection

Ninety-five field serum samples were tested with both the *N*-ELISA assay and a commercial ELISA kit (IDEXX). The IDEXX kit could detect antibodies against various structural proteins of IBV while the *N*-ELISA detected only specific an-

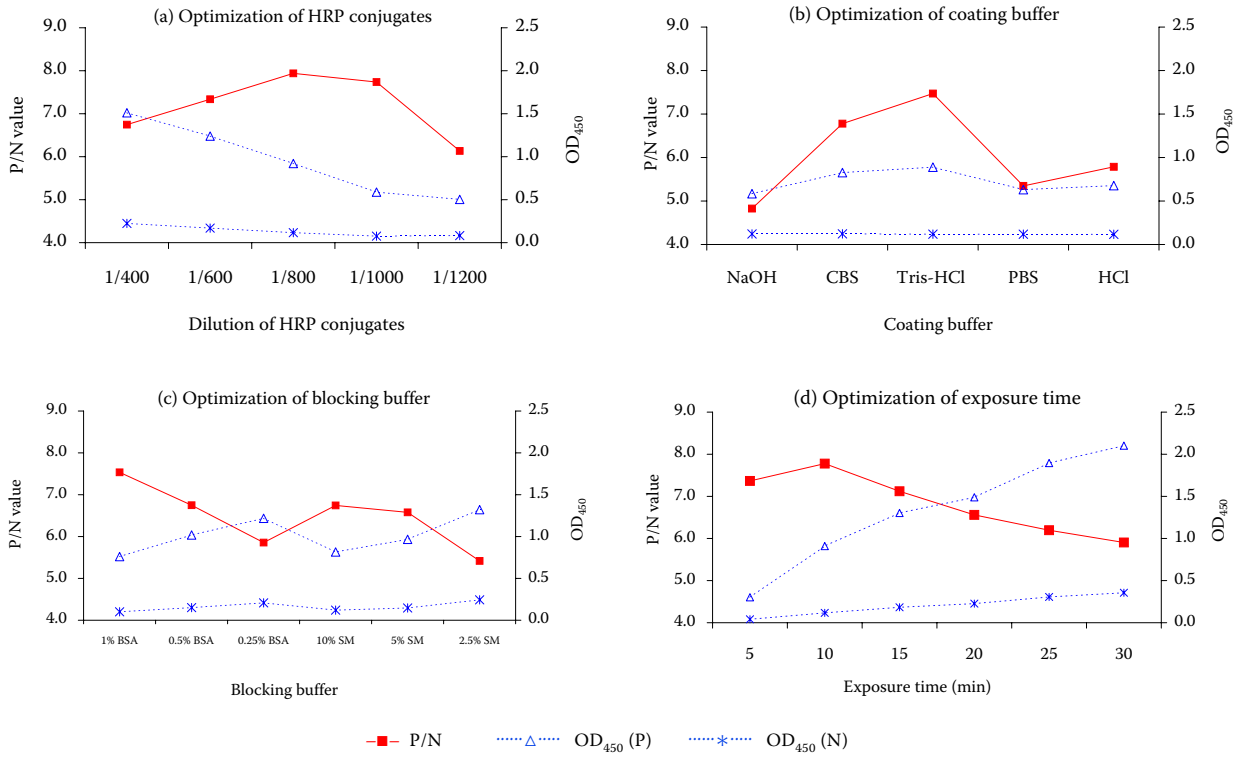

Figure 4. Optimization of HRP conjugates dilution, coating buffer, blocking buffer, and exposure time. (a) Optimization of dilution of conjugates (HRP labeled rabbit anti-chicken IgG). (b) Optimization of coating buffers: NaOH (pH13), 0.05M Bicarbonate/Carbonate buffer (CBS, pH 9.6), 0.1M Tris-HCl buffer (pH 8.5), Phosphate buffer saline (PBS, pH 7.4) and HCl (pH 4.6). (c) Optimization of blocking buffer: BSA, SM. (d) Optimization of exposure time

tibodies against N protein of IBV. The two methods showed a similar curve of antibody titers (Figure 5). The total number of positive samples detected by the *N*-ELISA assay using N protein and the commercial ELISA kit was 95% (90/95) and 99% (94/95), respectively. The *N*-ELISA assay shared 95.7% identity with the commercial ELISA assay in the total positive for antibodies to IBV. The results indicated that the antibody titers against N protein are closely correlated with those against full virions, and the *N*-ELISA assay was appropriate for evaluation of the immune status of chickens vaccinated against IB or exposed to IB virus. In addition, both of the ELISA assays showed that the antibody titer in Farm 3 is much lower than those in Farm 1 and Farm 2.

DISCUSSION

The N protein of IBV strains Gray, Beaudette and M41 has already been expressed in *E. coli*, insect cells or saccharomyces cerevisiae (Ndifuna et al., 1998; Chen et al., 2003; Gibertoni et al., 2005). It was found that the recombinant N protein was appropriate for immunoassay and N protein expressed in *E. coli* showed better immunogenicity than that expressed in insect cells (Chen et al., 2003). Previous studies concentrated on classical IBV strains which were isolated many years ago. IBV is highly variable and the epidemic nephropathogenic IBV strains in China currently shared low identity with classical strains (Zhou et al., 2003b, 2004b). In the present study, the nucleocapsid protein gene of IBV strain X isolated in China was successfully expressed in *E. coli* at a high level in the vector pBAD/his B. Meanwhile, by western blot analysis, we also demonstrated that the different molecular weight of the fusion N protein of IBV existed in the bacterial lysates, i.e., 50 kdD, 46 kd as well as several smaller polypeptides, indicating that the expressed N protein was degraded in host *E. coli*. Also, in western blot analysis, the degraded segments of the fusion N protein could be recognised with mouse anti-Xpress mAb, showing that the N terminus of the

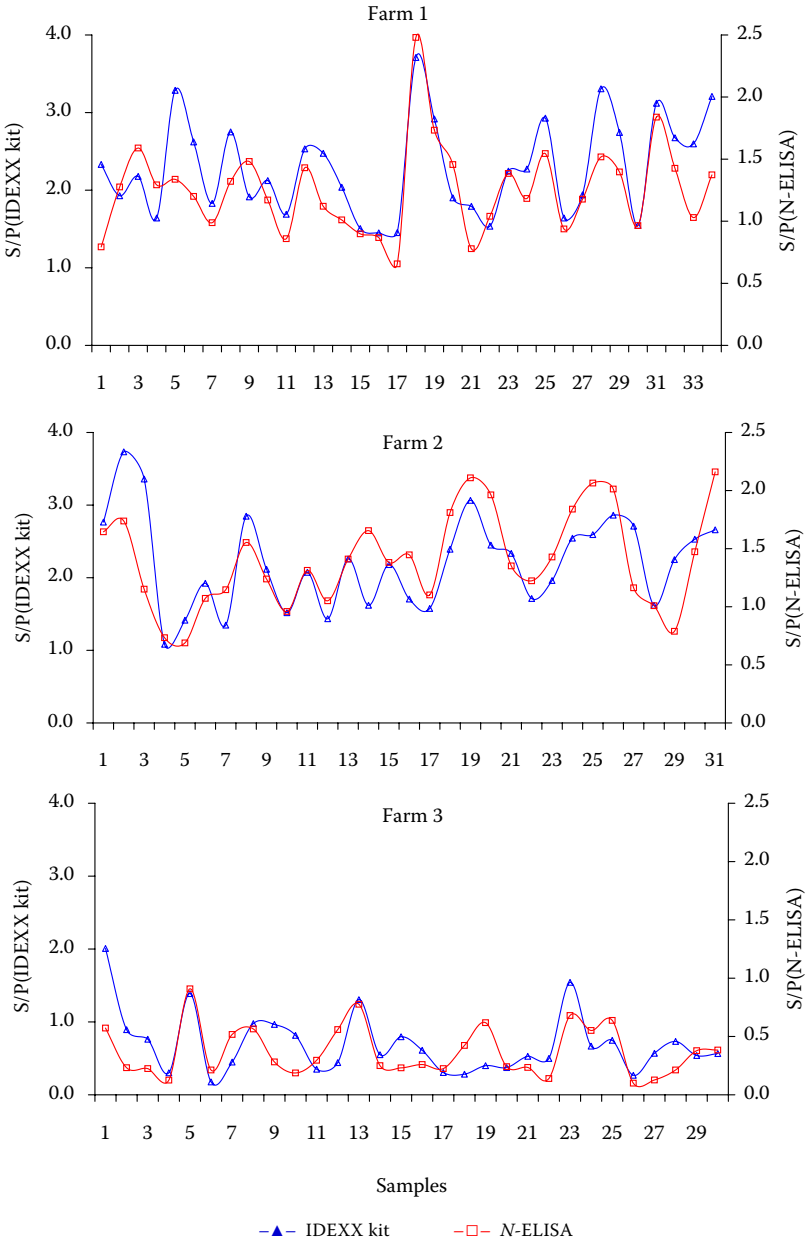

Figure 5. Antibody titer curve of field samples produced with both the ELISA assay using N protein (*N*-ELISA) and a commercial kit (IDEXX kit). Chickens in Farm 1 and Farm 2 were 140-day-old and 110-day-old white Leghorn chickens, respectively. Chickens in Farm 3 were 120-day-old Sanhuang chickens (a Chinese local breed). All of the chickens in the three farms were vaccinated with commercial vaccines (H120 and H52)

N protein was untouched, and that the N protein was readily disrupted near the C terminus where there might be protease recognizing sites. In this experiment, to prevent the degradation of the expressed N protein of IBV, the bacteria were cultured at 25°C and 30°C after induction. However, our attempts were unsuccessful, presuming that the degradation of the expressed products may be digested by the proteolytic in host bacteria.

Purification of the N protein is one of the critical steps for *N*-ELISA assay in the present study because the N protein of IBV was produced in *E. coli*. Poor purity would severely interfere with the immunoreaction since chicken flocks usually possess antibody against *E. coli*. Nickel column could specially combine with 6His tag at the N terminus of N protein for purification. The N protein could be purified with both native and denaturing protocols.

It was found that more N protein was produced with denatured protocol and the denatured N protein remained immunogenic. It might be that in the denatured protocol bacteria were lysed thoroughly and more proteins was released. When the N protein was denatured lots of inner epitopes was able to be exposed, which might contribute to the immunoreaction of N protein with antibodies. To evaluate the purity of N protein, western blotting was performed with antisera against *E. coli* and IBV. Shorter polypeptides were shown to be not protein of *E. coli* but partial segment of N protein this way.

The protocol for the *N*-ELISA was optimized in the present study because many factors might affect the results of the ELISA (Figure 4). As to the coating of N protein, the use of Tris-HCl at pH 8.5 conferred the most efficient coating condition despite the fact that bicarbonate/carbonate buffer at pH 9.6 (Figure 4b) is used widely in previous reports. As to the standard for judgment in ELISA, the standard usually is absorbency value, S/N ratio (absorbency value of sample/absorbency value of negative sample) or S/P ratio (absorbency value of sample/absorbency value of positive sample). In our experiment, S/P ratio was chosen because it was more stable than other standards. In fact, S/P ratio has also been chosen as standard in many previous reports (Wang et al., 2002; Chen et al., 2003) as well as in a commercial ELISA kit (IDEXX, Westbrook, Maine, USA).

The *N*-ELISA established in the present study showed that the N protein of IBV strain X was only able to react specifically with the antisera against various IBV (strains M41, H52, H120, X and ZJ971) while not with antisera against AIV, IBDV, NDV and *E. coli*. Subsequently, in a correlation experiment, the *N*-ELISA assay indicated 95.7% identity to the commercial ELISA assay in the total number positive for antibodies to IBV (Figure 5), demonstrating that the *N*-ELISA may be used as a potential technique for evaluation of the immune status of chickens vaccinated against IB or exposed to IB virus.

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