Establishment of an in-house, recombinant nucleocapsid protein-based enzyme linked immunosorbent assay (ELISA) for the detection of chicken infectious bronchitis

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Infectious bronchitis (IB) is one of the devastating chicken viral diseases that causes significant financial loses to chicken industry worldwide. In this study, highly purified recombinant nucleocapsid protein (rNP) of IB virus (IBV) was used to develop an in-house enzyme linked immunosorbent assay (iELISA) for the detection of IB. The iELISA was optimized to achieve maximum sensitivity and specificity. All tested broiler chicken farms were positive for IBV antibody in a commercial ELISA kit (cELISA) and the optimized iELISA. Thirty three (33) specific pathogen free (SPF) chickens and 20 broiler chickens held in a restricted environment tested negative for IBV antibody in both ELISA. The above results suggest that the iELISA is a sensitive and specific test for the detection of antibody to IBV in chicken flocks.

Key words: Enzyme linked immunosorbent assay (ELISA), infectious bronchitis (IB), recombinant nucleocapsid protein (rNP).

INTRODUCTION

Infectious bronchitis (IB), a highly contagious upper respiratory disease, was first reported in 1931 (Schalk and Hawn, 1931; Collisson et al., 1992; Cavanagh and Naqi, 2003) and continues to be one the major threats to chicken industry worldwide. The disease has been described in young and laying chickens with age-dependent clinical signs. In young chickens, common respiratory signs including tracheal rales, sneezing and coughing are observed. The virus may also affect non-respiratory organs including kidneys and oviducts of young chickens (Darbyshire et al., 1979). IBV-induced clinical signs in adult laying chickens are poor egg quality and significant reduction in egg production (Ignjatovic and Sapats, 2000; Hofstad, 1984).

IBV (genus coronavirus, family Coronaviridae) (Cavanagh and Naqi, 2003) contains 27.6 kb single stranded positive sense RNA (Boursnell et al., 1987). Many serotypes have been reported for IBV (Cavanagh, 2007; Ignjatovic and Sapats, 2000). The genome encodes three major structural proteins; the 150 to 200 kDa spike (S) glycoprotein, the 20 to 30 kDa integral membrane glycoprotein (M), and the 43 to 50 kDa nucleocapsid (N) phosphoprotein (Cavanagh, 1981; Spaan et al., 1988; Saif, 1993). The immunodominant N protein (NP) induces cross-reactive antibodies (Ignjatovic and Galli, 1994) and enhances the protection level when it is expressed endogenously (Tang et al., 2008; Yang et al., 2009). The recombinant full length NP of IBV gray strain
has been used to develop an enzyme linked immunosorbent assay, ELISA (Ndifu et al., 1998). Also, partial NP has been utilized for the development of an IBV ELISA (Lugovskaya et al., 2006).

IBV infects chicken flocks worldwide. The disease has been reported from chicken flocks in Iran using conventional serological and virological methods (Aghakhan et al., 1994; Momayez et al., 2002; Vasfi and Bozorgmehri, 2000). Using molecular techniques, we and other research groups have detected and differentiated Iranian IBV isolates (Ghahremani et al., 2011). Despite the extensive use of IBV live attenuated and inactivated vaccines to protect commercial chicken flocks in Iran, IB remains responsible for serious financial losses to the poultry industry of the country. This study tested whether the full length recombinant NP (rNP) derived from the H120 strain of IBV can be used to develop an ELISA for the detection of IBV antibody.

MATERIALS AND METHODS

Recombinant IBV N protein

Expression of the recombinant IBV NP (rNP-IBV) was performed using a bacterial expression system as described previously (Haqshenas et al., 2004). Briefly, the full length N gene of IBV strain H120 was reverse transcription-polymerase chain reaction (RT-PCR) amplified and cloned into the pET-23a(+) plasmid. The final construct was named pET-23a-NP. To prepare the recombinant N protein for ELISA, Escherichia coli BL21(D3) bacteria were transfected with the pET-23a-NP construct and grown using Lautryl broth (LB) medium supplied with 50 µg/ml of ampicillin. The expression of protein was induced using isopropyl-β-D-thiogalactopyranoside (IPTG) at 30°C overnight. The rNP contained a six-histidine tag at its carboxyl terminus (C-terminus) allowing the purity of the protein by affinity chromatography using Ni+ resin as previously described (Haqshenas et al., 2004). Following elution of the protein in the elution buffer (50 mM NaH2PO4, 300 mM NaCl; pH 8.0) containing 250 mM imidazole, the protein was quantified using the Bradford method (Bradford, 1976). The aliquots of the purified protein were stored at -70°C and the required amounts were thawed for the performance of the optimization experiment and the detection of IBV antibody.

Serum samples

Two hundreds and twenty seven (227) serum samples were received from 21 broiler chicken farms by Iranian Veterinary Organization (IVO), Tehran. Thirty three (33) serum samples were collected from specific pathogen free (SPF) chickens reared in specific isolators located at the central laboratory of the IVO. Twenty samples were collected from 15-day-old broiler chickens held in a restricted environment. All above samples were tested for IBV antibody using the well established whole virus-based IDEXX IBV commercial ELISA kit (cELISA) (IDEXX, Westbrook, ME) by IVO according to the manufacturer's instructions.

Optimization of iELISA

To achieve the maximum sensitivity and specificity for iELISA, several factors were optimized. The amount of antigen per each well is critical for the optimal detection of antibody. To determine the optimal quantities of antigen, the rNP purified by affinity chromatography (Haqshenas et al., 2004) was diluted in carbonate buffer (pH 9.6) to final concentrations 0.25, 0.5, 1, 2, 4 and 8 µg/100 µl buffer. 100 µl of the above antigen preparations were added to each well of 96-well plate (Nunc). The plates were incubated at 37°C for 2 h. The unbound antigen was removed by one wash with PBS. The unfilled areas were blocked using blocking reagent (5% skimmed milk, 4% rabbit serum in PBS, pH 7.4) overnight at 4°C. The coated wells were washed twice with PBS-T (0.05% Tween-20 in PBS, pH 7.4) and twice with PBS. 100 µl IBV-positive primary antibody (Haqshenas et al., 2013) diluted in 0.5x blocking reagent (1/400) were added to each well. Following 30 min at 37°C, wells were washed as above. The developing solution containing 2,2'-azinodi(3-ethyl benzthiazoline sulfonic acid) (ABTS) chromogen (Sigma) in citrate buffer (pH 4.2) and 0.1% H2O2 were added according to the manufacturer's instructions. The plates were incubated for 15 min at room temperature before the absorbance values were measured at 405 nm using an ELISA reader. To determine the optimal concentration of primary antibody, serial two-fold dilutions (1/50 to 1/102400) of the positive serum (Haqshenas et al., 2004) were tested. One microgram antigen was used to coat each well and ELISA was performed as described above. To determine the optimal concentration of secondary antibody, it was serially two-fold diluted (1/250-1/512,000). ELISA was formed as above using 1 µg antigen per well and primary antibody was diluted 1/400. Throughout this study, a control negative excluding primary antibody was included as the background control and the obtained OD value was subtracted from the positive and negative OD values.

Examination of clinical serum samples for IBV antibody

Serum samples received from IVO were diluted in 5x blocking reagent to a final concentration of 1/400 and tested in duplicate. Using 1 µg antigen per well and secondary antibody at dilution 1/500, iELISA was performed as described above. A conservative cutoff value of 0.16 (mean of OD values of negative samples, 0.07, plus six standard deviations, 0.015) was set as described previously (Haung et al., 2002). The OD values below and above the cutoff were considered negative and positive, respectively.

RESULTS AND DISCUSSION

Optimization of iELISA

Optimization of ELISA for the achievement of maximum specificity and sensitivity using minimum quantities of reagents is a critical step in the establishment of a new ELISA. To optimize iELISA, optimal concentrations of several reagents including antigen, primary and secondary antibodies were determined. We aimed to produce a signal not exceeding 1 OD. To identify the best concentration of antigen per well, the highly purified rNP was prepared using affinity chromatography as described (Haqshenas et al., 2004). SDS-PAGE and immunoblot analysis revealed that in consistent with our previous report (Haqshenas et al., 2004) the antigen preparation was highly pure (data not shown). A wide range of antigen quantities (0.25 to 8 µg per well) were utilized for the determination of the best antigen concentration per well.
Figure 1. Optimization of in-house ELISA. (A) Various concentrations of the recombinant IBV N protein (rNP-IBV) were used for the optimization of antigen concentration. (B) The IBV-positive serum sample (Haqshenas et al., 2013) was serially two-fold diluted and tested using 1 µg antigen per well and 1/500 secondary hP-conjugated antibody. (C) Using 1 µg antigen per well and primary antibody at dilution 1/400, serially two-fold diluted secondary antibody was tested. iELISA was performed as described in Materials and Method.

As can be seen from Figure 1, 1 µg antigen per well produced ~1 OD (Figure 1A) and represented the optimal antigen concentration for the performance of iELISA. The background OD value using negative serum sample remained the same for all antigen quantities (Figure 1A). Subsequently, the best primary and secondary antibody dilutions were determined to be 1/400 (Figure 1B) and 1/5000 (Figure 1C), respectively. The above dilutions produced approximately 1 OD value and the background signal did not exceed ones obtained using other dilutions.

Detection of antibody to IBV in clinical serum samples

ELISA is the most economical and convenient test for the examination of large numbers of clinical serum samples for the presence of antibody. Several cELISA kits are available from different companies. However, the commercial kits are expensive and some circumstances may disrupt their export and import. We have successfully developed an ELISA using bacterially expressed avian hepatitis E virus (HEV) ORF2 protein for the detection of avian HEV antibody (Haung et al., 2002). Recently, we developed a highly sensitive and specific rNP-IBV based dot-blot assay for the detection of antibody to chicken IBV in clinical serum samples (Haqshenas et al., 2013). The current study was designed to use the IBV rNP to develop an iELISA kit for the serological detection of IB disease and the immune response status to the IBV vaccination. The NP was used in this assay because it is highly conserved among IBV strains with 94 to 99% identity (Williams et al., 1992; Sneed et al., 1989). Therefore, the rNP based iELISA can be potentially used to serologically identify chickens infected with any IBV strain although further experimental data are required to confirm this point. Following optimization, to evaluate the sensitivity and specificity of iELISA, 227 clinical serum samples from 21 chicken farms were examined for IBV antibody using the cELISA and iELISA. All 21 tested chicken farms (227 serum samples) were found to possess antibody to IBV antibody in both ELISA suggesting 100% sensitivity and specificity of the iELISA in the detection of IBV-antibody positive chicken flocks. The above results suggest a high correlation between the two ELISAs (100% agreement). In addition, the results of iELISA and cELISA on the individual 227 serum samples were compared. Using the cELISA, 204 and 23 serum samples tested positive and negative for the presence of IBV antibody, respectively. In the iELISA, 209 and 18 sera were positive and negative, respectively. Seven out
of the 23 samples negative in the cELISA produced OD values above the cutoff in the iELISA. These seven samples were also positive for IBV antibody when they were tested by two additional tests dot-blot and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)/immunoblot analyses (Haqshenas et al., 2013). SDS-PAGE/immunoblot analysis revealed that these sera specifically interacted with the IBV NP (Haqshenas et al., 2013). Therefore, including these seven samples, the apparent sensitivity of iELISA was higher than the cELISA (209 vs 204 out of 227 samples) and approximately 2% of the positive samples remained undetected in the cELISA. The failure of the cELISA to detect these seven samples might be due to the difference in the coating antigen that has been used by the kits; the cELISA uses the whole IBV while iELISA uses the rNP. These seven chickens might have seroconverted to the N protein more efficiently than to the outer proteins like the S glycoprotein. Consequently, these seven samples were detected in the iELISA but not in the cELISA. Of 204 positive samples in the cELISA, 202 samples produced OD values above the cutoff in the iELISA. This difference could be due to technical error or lower specificity of cELISA. Also, there is a possibility that these two chickens seroconverted to the other viral structural proteins more efficiently than to the N protein. Unfortunately, due to lack of access to the antigen used by the IDEXX Laboratories, it was impossible to investigate the specific interaction of antibody and IBV antigen. A summary of the results has been presented in Table 1. To test the specificity of the iELISA, 33 serum samples from SPF chickens and 20 serum samples collected from chickens held in a restricted environment were tested by both ELISA. All the tested samples were negative for IBV antibody using both ELISA. The above results suggest 100% specificity for the iELISA. Additionally, all IBV-negative serum samples containing high antibody titers to Newcastle disease virus (NDV), infectious bursal disease virus (IBDV), and influenza virus produced OD values below the cutoff further indicating high specificity of iELISA. In conclusion, the above results demonstrate that iELISA is a specific and sensitive test for the detection of IBV antibody and can be widely used as a substitution for the commercial kits.

Table 1. 227 serum samples from 21 chicken farms were tested by the commercial ELISA (cELISA) and in-house ELISA (iELISA).

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<th>Pos</th>
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Pos, Positive; Neg, negative.

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REFERENCES


