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Evaluation of four enzyme linked immunosorbent assays for the detection of antibodies to infectious bursal disease in chickens

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A B S T R A C T

The routine technique for detecting antibodies specific to infectious bursal disease virus is a serological evaluation by enzyme linked immunosorbent assay (ELISA) with preparations of whole virions as antigens. To avoid the use of complete virus in the standard technique, in-house VP2 and VP3 based ELISAs were developed. Accordingly, four types of indirect ELISAs *viz*., a commercial IDEXX-ELISA kit, VP2 and or VP3 antigen based ELISAs and a whole virus ELISA were compared with the virus neutralization test. It was concluded that the sensitivity and specificity at receiver-operating characteristics (ROC) optimized cut-off of four ELISAs *viz*., IDEXX-ELISA, VP2-ELISA and VP3-ELISA indicated similar performance whereas whole virus antigen based ELISA showed poor performance in comparison to other ELISAs. Similarly the positive and negative likelihood ratio of four ELISAs at an optimized cut-off indicated IDEXX-ELISA to be the best among all the four ELISAs while the performance of rVP3-ELISA and rVP2-ELISA is good as compared to the whole virus ELISA. Finally, the area under the ROC curve (AUC) of four ELISAs which represented a summary statistics of the overall diagnostic performance of the test also indicated that the IDEXX-ELISA, VP3-ELISA and VP2-ELISA had similar and relatively better performance when compared to whole virus antigen-ELISA.

1. Introduction

Infectious bursal disease, a highly contagious viral disease of young chicken is caused by infectious bursal disease virus (IBDV) (Giambrone et al., 1977) and characterized by immunosuppression and mortality, generally at 3–6 weeks of age. IBDV infects the bursa of Fabricius, particularly the actively dividing and differentiating lymphocytes of the B cells lineage of young chickens, resulting in morbidity and immunosuppression (Saif, 1991). The viral genome consists of two segments of double stranded RNA *i.e.* segment A (3.2 kb) and segment B (2.8 kb) (Hudson et al., 1986). Segment A encodes 110 kDa polyprotein that is auto proteolytically cleaved to pVP2, VP3 and VP4. The pVP2 is further processed to VP2 (Lombardo et al., 1999; Muller and Becht, 1982). VP2 and VP3 are the major structural proteins of the virus (Dobos et al., 1979). Recombinant VP2 protein has been developed for detection of antibodies against IBDV in *Escherichia coli* (Fahey et al., 1989), yeast (Pitcovski et al., 2003; Dey et al., 2009) and insect cells (Lee et al., 2006a, 2006b). Subsequently it was found that VP3 is a group specific major immunogenic protein of IBDV. After infection with live or inactivated IBDV, the earliest appearing antibodies are directed towards VP3 (Fahey et al., 1985).

Serological diagnosis is carried out usually by agar gel immunodiffusion test (Hirai et al., 1974; Jeon et al., 2008), virus neutralization test (Weisman and Hitchner, 1978) or enzyme linked immunosorbant assay (ELISA) (Howie and Thorsen, 1981; Jeon et al., 2008). Agar gel immunodiffusion is the simplest test, but its sensitivity and specificity may vary among laboratories and is time consuming. Virus neutralization has the highest specificity and correlated with protection. But as virus neutralization is labour intensive, requires the facilities of a virology laboratory and provides delayed results, it is impractical for routine use. ELISA is the assay used most commonly as it is sensitive, specific and quantitative. Commercial ELISA kits are available to detect antibodies to IBDV in field sera samples. Recombinant IBDV proteins such as VP2 (Jackwood et al., 1996; Martínez-Torrecuadrada et al., 2000; Dey et al., 2009) and VP3 (Wang et al., 2008) can detect IBDV-specific antibodies. The use of virus as a diagnostic antigen in the conventional tests warrants careful handling of the virus, which may otherwise lead to outbreaks of infection. Recombinant IBDV proteins are safe, non-infectious, highly specific and immunogenic. The sensitivity and specificity of IBDV-specific detection using pVP2 expressed in insect cells are better than those of using insect cell derived VP3 protein (Martínez-Torrecuadrada et al., 2000). The present study

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evaluated the specificity and sensitivity of a recombinant VP2 and VP3 antigen based ELISA developed "in-house" with other conventional methods for the detection of IBDV infection.

2. Materials and methods

2.1. Expression and purification of recombinant VP3 (rVP3) protein

Viral RNA was extracted from chicken embryo fibroblast cells infected with IBDV using Trizol (Sigma, St. Louis, USA) according to manufacturer's instructions. Reverse transcription was carried out with the extracted RNA using the Thermoscript RT kit (Invitrogen, Carlsbad, USA) to synthesize the first strand cDNA. Oligonucleotide primers were synthesized for amplifying the full length VP3 coding gene, corresponding to 774 bp with incorporated restriction sites for directional cloning. The sequence of the primers is as follows:

Forward: 5′CCCGAATTCATGGCATCAGAGTTCAAAGAGACCCCTG3′
Reverse: 5′CCCGCGAACGGATCCAATTT3′

The PCR amplicon was cloned directionally into the bacterial expression vector pET32a(+) and transformed into *E. coli* BL21 (DE3) pLysS. The nucleotide sequence of the RT-PCR amplified gene was submitted to Gen Bank under the accession number EU621836. Isopropyl-B-D-thiogalactopyranoside (IPTG; 1 mM final concentration, MBI Fermentas, St.Leon-Rot, Germany) was added to log phase cultures of BL21 (DE3) pLysS cells transformed with pET32a(+) recombinant plasmid for expression of His6 fusion recombinant VP3 protein. The culture pellet containing the recombinant protein was solubilized with 8 M urea. The recombinant 6× histidine tagged VP3 fusion protein was purified by affinity chromatography containing Ni-NTA (Invitrogen, Carlsbad, USA). The recombinant protein was purified further using the Amicon ultra-4 centrifuge filter device (Millipore, Bedford, USA) with a membrane cut-off of 30 kDa. The concentration of the purified protein was determined by Lowry's method (Modified Lowry's Protein assay kit) according to the manufacturer's protocol (Pierce, Rockford, USA). The confirmation and purity of the recombinant VP3 protein was analyzed by SDS-PAGE and Western blotting using anti-His antibody (Qiagen, Hilden, Germany).

2.2. Enzyme linked immunosorbent assay with the recombinant VP2/VP3, IDEXX-ELISA kit and with whole virus antigen

2.2.1. Recombinant VP2, VP3 and whole virus antigens

In this study, VP2 produced in yeast (Dey et al., 2009) about 20 nm diameter was used for VP2-ELISA. The vaccine virus strain (Georgia) was used as the whole virus antigen for coating the ELISA plate.

In an indirect ELISA format, recombinant VP2, VP3 and the whole virus were coated on to flat bottom 96-well plates (Nunc, Roskilde, Denmark) and the plates stored at 4 °C overnight. The optimal antigen concentration of recombinant VP2 or VP3 was determined by checker board titration (Dey et al., 2004). The optimum antigen concentration of whole virus antigen was determined at different dilutions ranging from 1:50 to 1:500. Plates were incubated with blocking solution in phosphate buffered saline (PBS) supplemented with 0.05% Tween 20 (PBST) and 2% (w/v) bovine serum albumin for 1 h at 37 °C, washed three times with PBST and stored at −20 °C until use.

2.2.2. The IDEXX-ELISA kit

The anti-IBDV antibody titre was also determined by a commercial antibody ELISA kit for IBDV (IDEXX Laboratories, Westbrook, USA) according to manufacturer's instructions. Serum samples with an S/P ratio of less than or equal to 0.2 were considered negative.

2.2.3. Test sera

A total of 151 chicken serum samples were screened. Chicken blood samples were collected from the Central Avian Research Institute, Izatnagar, India. Blood samples were collected under aseptic conditions and then centrifuged at 1500 × *g* for 20 min.

2.2.4. Peroxidase conjugate

A volume of 100 μl of rabbit anti-chicken IgG peroxidase conjugate (Sigma, St. Louis, USA) diluted 1:3000 was added to each test and to the control wells. The concentration of anti-chicken IgG peroxidase conjugate was determined by checker board titration.

2.2.5. Substrate

O-phenylenediamine dihydrochloride (OPD) (Sigma, St. Louis, USA) 1 mg/ml in 0.1 M citrate phosphate buffer (pH 5.0) mixed with 1 μl/ml of 35% hydrogen peroxide was used. The substrate, 100 μl/well was dispensed immediately into the wells and incubated until the colour reaction developed. The plates were read at 492 nm in an ELISA reader (Biotek Instruments Inc., Winooski, USA).

2.3. Virus neutralization test

The serum samples were inactivated at 56 °C for 30 min. Then they were diluted two fold, starting at a 1:2 dilution, in GMEM and mixed with 50 μl of 100 TCID50 of a local IBDV isolate BS07 per well (final serum dilution, 1:4096). After 1 h at 37 °C, the antibody–virus mixture was added to 1×10^4 CEF cells in GMEM and 10% FCS per well of a 96-well culture plate (Costar, Corning, Ithaca, USA). After incubation at 37 °C for 4 days, cell monolayers were washed with PBS and stained with haematoxylin and eosin stain. The virus neutralization titre of antibodies in a serum sample was determined as the reciprocal value of the highest serum dilution that causes a 50% reduction of the cell monolayer.

2.4. ROC analysis

Receiver-operating characteristic (ROC) curves, the area under the curve (AUC), test predictive values (+/−), likelihood ratios (+/−) and determination of optimal cut-off points (Greiner et al., 2000) were conducted with the software MedCalc 9.2.

3. Results

3.1. Expression of recombinant VP3 protein

The full length VP3 gene was cloned and expressed from pET32a(+) as a fusion protein with 6× His tag and was about 48 kDa (Figs. 1 and 2) in size and the average yield of the recombinant protein was 0.6 mg/ml of culture.

3.2. Evaluation of virus neutralization test

A total of 151 field serum samples were tested by the virus neutralization test, of which 77 and 74 serum samples were found to be seropositive and seronegative respectively.

3.3. Enzyme linked immunosorbent assay with recombinant VP2/VP3 and whole virus antigen

An indirect ELISA was carried out to determine the feasibility of recombinant proteins VP3 or VP2 as diagnostic reagents for IBDV.