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Development of Single Serum ELISA and Flow Through Assay for Infectious Bursal Disease of Poultry

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Abstract

The infectious bursal disease (IBD) is an age-limiting viral disease of chicken affecting both broiler and layer chicks between 3 – 6 weeks of age characterized by severe immunosuppression and high mortality. The maternally derived antibodies protect chicks till they develop age – resistance, hence breeder flocks immune status monitoring regularly helps in ensuring adequate levels of maternal antibodies transfer to hatchlings and fine tuning of vaccination schedule. The conventional virus neutralization test (VNT) though gold standard, is time consuming and cumbersome. Hence, alternate immunodiagnostic tests which are simple and relatively easy to perform viz., single serum dilution enzyme linked immunosorbent assay (ELISA) for antibody titre and flow through assay (FTA) for antigen detection were developed. A standard curve was constructed by using regression analysis which helped in derivation of an equation and that allowed to demonstrate correlation between observed titre and predicted titre. At a dilution of 1:2000 of serum there was a linear relationship between predicted titres at single serum dilution and observed titre. The FTA was able to detect 200 ng / µl concentrations of IBDV. The FTA can be performed as, on spot test for detection of IBDV in suspected cases.

Keywords: Infectious bursal disease virus; Immunodiagnostics ; Single serum dilution ELISA; Flow through assay

Introduction

Infectious bursal disease virus (IBDV) is an important immunosuppressive and re-emerging viral disease of poultry, paving path for so many other pathogenic infections. IBDV infection causes impaired immune response to many viral infections like Newcastle disease virus (Allan *et al*., 1972; Faragher *et al*., 1974), making the chickens highly susceptible to other viral infections like inclusion body hepatitis (Fadley *et al*., 1976), Marek's disease (Cho, 1970; Sharma, 1984), infectious bronchitis (Pejkovski *et al*., 1979), infectious laryngotracheitis (Rosenberger and Gelb, 1978), chicken anaemia virus (Yuasa *et al.*, 1980) and reoviruses (Moradian *et al*., 1990). Along with viral infections, IBDV infection also increases susceptibility to secondary bacterial infections like *S. typhimurium* and *E. coli* (Wyeth, 1975), S.aureus (Santivatr *et al*., 1981), and coccidial infections (Anderson *et al*., 1977; Onaga *et al*., 1989). Infectious bursal disease (IBD) causes high mortality in 3 to 6 week old chicken, whereas less acute or subclinical disease is common in 0 to 3 week old chicken (OIE manual, 2004) and IBDV is immunosuppressive at an early age. The monitoring of health status of the flock at regular intervals definitely curtails IBDV infection. Among various indicators of health monitoring, checking immunization status helps to protect flocks optimally and improving the vaccination programme. This monitoring has to be done on a permanent basis (Veielitz, 1997). The chicks with adequate maternal antibodies will combat the infection till they develop age – resistance. The effective transfer of maternal antibodies to chicks can be achieved by proper breeder flock hyper immunization. Hence, the immune status of breeder flocks is to be monitored periodically on a regular basis. The fine-tuning of breeder flock vaccination and of hatch-

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lings can be achieved by screening with serological tests. The conventional virus neutralization test, though gold standard, time consuming; cumbersome and requires sophisticated laboratory facilities. Screening of poultry flocks with sensitive, rapid and reliable immunoassays like ELISA helps in monitoring immune status of flocks and large number of samples can be processed at ease. However, ELISA is more convenient, if the serum sample is subjected to a particular dilution rather than performing multiple dilutions if large flocks are to be screened. At the same time, for detection of IBDV antigen agar gel immunodiffusion test is time consuming and less sensitive. For this reason, a sensitive and user friendly FTA was designed and developed for IBDV detection which can be performed at farm premises even by semiskilled persons and helps to monitor infection status.

Materials and methods

Virus

The IBDV used was an isolate from a field outbreak in the Namakkal poultry belt of Tamil Nadu state, India. For the confirmation of virus, the bursal samples were processed as per the method described by Thangavelu (1996). Bursae collected from disease outbreak were made into 50% (w/v) suspension in PBS using sterile sand and the homogenate was centrifuged at low speed for 20 minutes. The supernatants were collected and extracted with chloroform as described by Dash *et al*. (1991). One volume of chloroform was added to 4 volumes of supernatant, mixed well, left at room temperature for 10 minutes and were centrifuged at 3000x g for 20 minutes. The clear aqueous phase was harvested, treated with penicillin (5000 I.U/ml), streptomycin (5000 µg/ml) and incubated for one hour at 370C. The sample was tested for antigen by agar gel immunodiffusion test (AGID) and counter immunoelectrophoresis (CIE) with IBDV antiserum obtained from Vaccine Research Centre – Viral Vaccines, Madhavaram, Tamil Nadu Veterinary and Animal Sciences University, Chennai, India and stored in small aliquots at -70^{0}C until further use.

Isolation of virus

The bursal sample which was found to be positive for IBDV antigen both by AGID and CIE was made into 10 % w/v suspension with sterile PBS and was used for virus isolation in chicken embryo fibroblast (CEF) cell culture.

Purification and concentration of virus

The IBDV was purified and concentrated as per the method described by Dobos *et al*. (1979) with few modifications. The clarified cell culture medium was treated with 2.2% (w/v) sodium chloride and 5% polyethylene glycol (MW 20,000). The mixture was stirred at 4^{0}C for 4 hrs. The precipitated material was collected after centrifugation at 40,000 g for one hour at 4^{0}C. The supernatant was discarded and the pellet was suspended in 10 ml of Tris – NaCl – EDTA (TNE) buffer. An equal volume of Freon (Chlorofluorocarbon, M/s SRL, India) was added and the aqueous and organic phases were separated by centrifugation at 40,000 g for 20 min at 4^{0}C. The aqueous phase was collected and layered onto two step caesium chloride gradient (1.2 g and 1.4 g / ml density) prepared in TNE buffer. This preparation was centrifuged at 132,000 g for 4 hrs at 4^{0}C using Beckman Ti60 rotor. The ultracentrifuge tubes were illuminated in a dark room and the viral band located between two caesium chloride gradient was collected.

Characterization of viral proteins

SDS-PAGE was performed as described by Laemmli (1970) to know the different viral proteins based on molecular weight. The purified and concentrated IBDV was quantified by the following formula which gives directly mg/ml (Wilson and Walker, 2007).
Protein (mg/ml) = OD at 280 nm x 1.55 – OD at 260 nm x 0.77.

Hyperimmune serum

The Institutional Animal Ethics Committee (IAEC) permission was granted for raising hyperimmune serum in chicken and rabbits. During the entire period of study, the laboratory animals were kept in good environment with *ad libitum* feed and water.

In chickens

For the production of hyperimmune serum in chickens, purified IBDV was used. A group of 6

birds were inoculated at 3-4 weeks of age by intranasal and intraocular routes with approximately 0.0005 g of purified virus per bird. A group of four birds were kept as control group without any inoculation. After 17 days of primary injection the first booster dose was given approximately 0.0015 g of purified virus per bird and a second booster dose was given 14 days after 1st booster. Two weeks after last injection the birds were test bled and confirmed the presence of antibodies by both AGID and CIE. Later large amount of blood was collected from birds and serum separated and stored in small aliquots at -20^{0}C until further use.

In rabbits

For the production of hyperimmune serum in rabbits approximately a 1 mg/ml quantity of purified IBDV was suspended in PBS and mixed thoroughly with an equal volume of Freund's complete adjuvant (Sigma, USA). Then, the total quantity was given into rabbit at different places subcutaneously. After one week of first dose, a second dose was given in the same manner but after mixing with the Freund's incomplete adjuvant (Sigma, USA). The rabbits were test bled after giving second booster and tested by AGID for precipitation line. After observing precipitation line in AGID, the rabbit was bled and the serum separated and stored in small aliquots at -20^0 C for further use.

Enzyme linked immunosorbent assay for antibodies

The optimum working dilutions of antigen and conjugate to be used in ELISA were arrived by performing checker board titration (CBT) as per the method described by Rose *et al*. (1997). The ELISA for the detection of IBDV antibodies was performed with modifications as described by Marquardt *et al*. (1980). The wells in polysterene microtitre plates were coated with 100 µl quantity of 2.5 ng/µl concentration of stock antigen in carbonate / bicarbonate coating buffer, pH 9.6. The plates were incubated overnight at 4^{0}C in refrigerator. After overnight incubation, the plates were washed three times with phosphate buffered saline Tween – 20 (PBS-T) and each well was added with 100 µl of blocking buffer and incubated at 37^{0}C for one hour. The plates were washed with PBS-T three times and 100 µl of each serum dilution was added to wells and the plates were incubated for 1 hour at 37^{0}C. The plates were washed with PBS-T again for three times. Then, 100 µl of Anitichicken HRP conjugate was added to all wells and incubated for 1 hour at 37^{0}C. The unreacted conjugate was washed again with PBS-T for three times and finally 100 µl of freshly prepared substrate (2, 2'–Azino–bis–3 ethyl benzthiazoline 6 – sulfonic acid, Sigma) prepared in substrate buffer was added in subdued light. The plates were incubated in the dark for 15 to 30 minutes at 37^{0}C for colour development. The colour reaction was stopped by adding 100 µl of 1% SDS in distilled water. The optical density (OD) readings were taken at 415 nm, using the ELISA reader.

Estimation of positive negative threshold (PNT) line

The positive negative threshold line was carried out as described by Snyder *et al.* (1983). The procedure mentioned briefly, ten serum samples, which were negative by AGID, were selected to perform ELISA. The serial dilutions of the serum were made from 500, 1000, 2000, 5000, 10000, 50000 and 100000. The resultant OD values were plotted on Y-axis against dilutions in X-axis. The resultant line is referred as PNT line and used for finding out the observed titre (OT).

Estimation of observed titre (OT)

The OT of 72 serum samples was calculated using the subtraction method (OD value for sample – OD value of conjugate control) as specified by Snyder *et al.* (1983) using the PNT line. Serial dilutions of the serum samples were carried out and ELISA was performed with these samples. The resultant OD values were plotted on Y-axis against dilutions in X-axis. The point where the sample line cuts the PNT line was taken as titre of the sample.

Development of titre calculation formula

The formula to find out the titre from single serum dilution instead of serial logarithmic dilution was developed by applying the principle of linear regression. The procedure is as follows, briefly, OD values obtained for every logarithmic dilution were compared with OT and correlation coefficient was obtained. The dilution that gave maximum positive

correlation was selected for predicting the titre from that dilution. The other parameters like slope and intercept were arrived by plotting a scatter chart with OD values in Y-axis and OT in X-axis. The linear regression formula Y=ax+b is reversed as x=(y-b)/a to find out the log titre and antilog of titre is referred as predicted titre of the sample.

Single serum dilution

The log ELISA titres were arrived by applying the formula. Log10 titre = (Corrected absorbance + 0.059)/ 0.049 and the titre is = antilog (Log10 titre).

Flow through assay for IBDV detection

The flow through assay for the detection of IBDV antigen was performed as described by Wang *et al*. (2005) with modifications. The cellulose acetate membrane (M/s mdi, Ambala Cantt) was placed above the absorbent pads in a flow through module. One µl (200 ng/µl) of IBDV was placed in the middle and 1 µl of rabbit serum was placed in a corner which acted as control. The membrane was dried in an incubator at 37^0C for 1 hour. The membrane was added with 200 µl of wash buffer and allowed to be absorbed through the membrane. This was repeated once again. Then the 200 µl of anti-rabbit serum diluted in wash buffer (1:10) was added and allowed to be absorbed through the membrane. The membrane was washed with wash buffer two times. Then the 200 µl of wash buffer diluted (1:2) protein A colloidal gold conjugate was added and allowed to be absorbed through the membrane. The wash cycle was repeated. Appearance of pink color dot indicated presence of antigen.

Results

Isolation

The IBDV obtained from embryo passages by chorioallantoic membrane route was again passaged four times in CEF cultures. The characteristic cytopathic effects like rounding and grouping of cells with cytoplasmic vacuolation were noticed in the first passage itself.

Characterization of viral proteins

The purified IBDV when subjected to protein analysis by SDS-PAGE revealed various polypeptides of the virus. After staining and destaining the gel with coomassie brilliant blue, the viral protein bands were identified. The IBDV revealed five structural polypeptides with four prominent bands viz., with molecular weights of 91, 000 Da (VP1), , 39,000 Da (VP2), 32,000 Da (VP3), 27,500 Da (VP4) and one light band with 49,000 Da (VPX) in SDS PAGE (Fig. 1). The total protein concentration of purified virus sample was 5.73 mg/ml.

Enzyme linked immunosorbent assay for antibodies

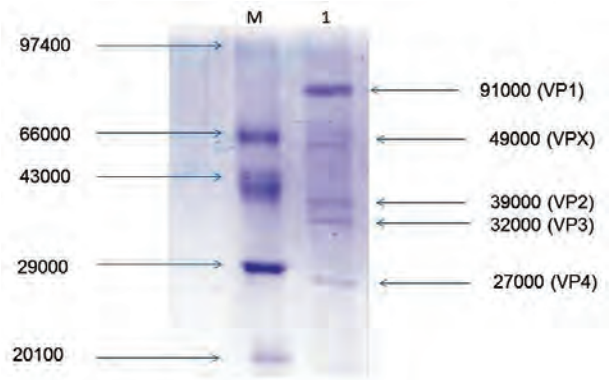

Fig.1. Protein profile of Infectious bursal disease virus: SDS-PAGE. Lane M: Molecular weight Markers. Lane 1: Viral Polypeptides

The dynamic working range of antigen and conjugate by checker board titration was found to be 2.5 ng/µl and 1:4000 respectively. Indirect ELISA was standardized to predict titre from single serum dilution, instead of using multiple serum dilutions. The prediction was made through a formula developed following the principles of linear regression. For developing the formula to predict the titre 72 serum samples obtained from field were tested. The ELISA titres of the samples are referred as observed titres and these were calculated by subtraction method. To find out the observed titre (OT), positive negative threshold (PNT) line was developed with 10 negative samples (Fig. 2). The dilution at which a sample cuts PNT line was taken as titre and referred to as OT. The OT thus arrived was compared with OD values of sample taken at different dilutions viz., 500, 1000, 2000, 5000, 10000, 100000 and correlation coefficient was arrived for each comparison. The correlation coefficient at 1: 2000 was 0.87, which was more than that of all other dilutions. Hence, 1:2000 was preferred to predict the titre.

The other two constants required to predict titres from serum dilution of 1:2000 viz., slope (A) and intercept (B) were estimated by drawing a scatter chart (Fig. 3). The slope and intercept were 0.049

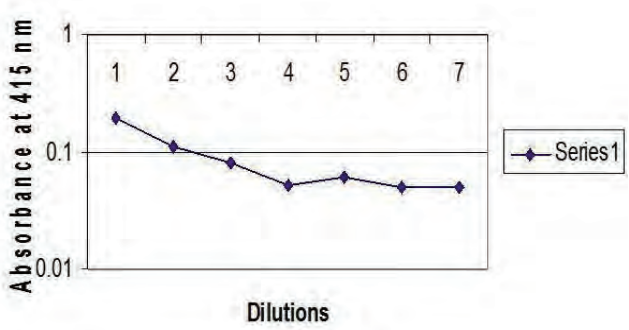

Fig. 2. Positive Negative Threshold line (PNT) for IBDV with IBD negative serum samples by AGID at dilutions viz., 1:500, 1000, 2000, 5000, 10000, 50000 and 100,000 on X-axis and on Y-axis corresponding OD readings. As the serum dilution increase the OD reading decreases. By using this PNT graph the observed titres of samples for formuala development were calculated.

and 0.059 respectively. Using these two constants, by applying the linear regression equation (y= ax+b), the formula for calculating titre was obtained which was read as follows x = (y-b) / a.
log 10 titre (x) = (Absorbance – intercept) / slope.

The observed and predicted titres of serum samples used to construct the formula were approximately equal (Fig. 4). After developing the prediction formula, a total of 302 serum samples

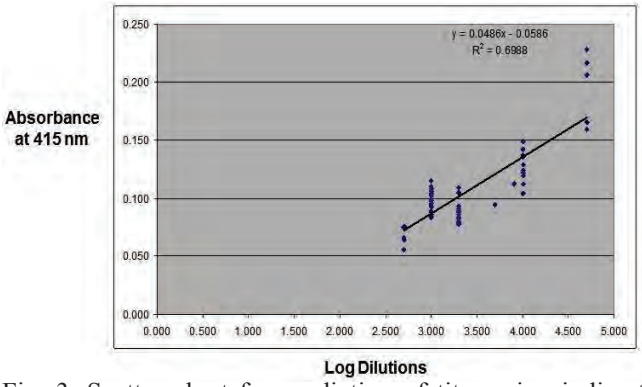

Fig. 3. Scatter chart for prediction of titre using indirect ELISA obtained from single serum dilution at 1:2000 dilution.

from broiler breeder flocks were screened for the presence of IBDV antibodies at 1: 2000 dilution of serum. The log titres were calculated using the developed formula and statistical parameters of measures of dispersion, namely, standard deviation and coefficient of variation (COV) were calculated. Samples, where coefficient of variation (COV) value exceeded 20% were not included for calculation and such samples were repeated.

Flow through assay for antigen detection

The flow through detected 200 ng/µl concentration

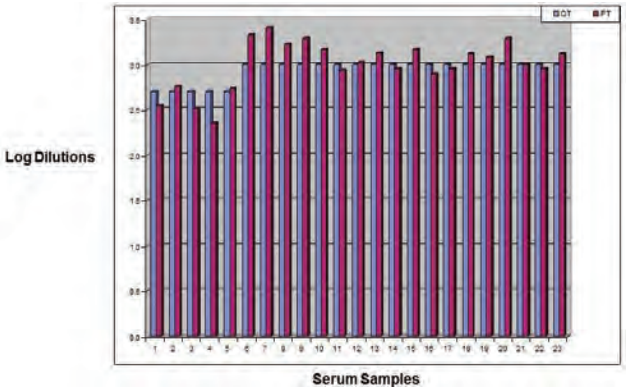

Fig. 4. Graph showing observed and predicted titres of different serum samples. The observed and predicted titres are approximately same.

of IBDV antigen and the dot developed in five minutes (Fig. 5).

Fig. 5. Flow through assay for IBDV antigen detection. Positive sample showing dot development with IBDV antigen (dot in the centre) and control antigen (dot in the periphery). Negative sample showing dot development only with control antigen (dot in the periphery).

Discussion

The present study was aimed at the development of alternate assays for detection of IBDV antigen and antibodies. Though VNT has been used as a gold standard for screening of IBDV antibodies, it is very time consuming and difficult to perform. In contrast, assays like ELISA and FTA are easy to perform, save reagent cost and time. The field isolate of IBDV that was used in the present study showed five structural polypeptides with molecular weights of 90000 Da (VP1), 40000 Da (VP2), 32000 Da (VP3), 28000 Da (VP4), 49000 Da (VPx) as detected in the SDS-PAGE corresponding to the expected size of IBDV proteins (Dobos *et al*., 1979; Muller and Becht, 1982). The ELISA has been applied to evaluate breeder flock immunity (Wyeth and Chettle, 1982). The practice of sequential sampling of flocks to monitor antibody level as influenced by vaccination, field exposure and time related decrease in titre were noted by emerging commercial ELISA kits. At the time of hatch, the birds were protected from the disease by maternal antibodies. As the chicks grew, protection by the

MDA decreases and birds became susceptible to IBDV infection. Vaccination to the flock was undertaken when there was decline of maternal antibodies, but not too low to contract the disease. This subtle line of differentiation is very important to schedule the vaccination or rescheduling vaccination to IBDV.

In the present study, the purified antigen was used to coat ELISA plates, which helped in avoiding non-specific reactions. The nonspecific reactions were generally noticed when the serum antibodies concentrations were too high. In high concentrations of serum the protein sticks to the plate and gives high background reading. To overcome these problems and also to evaluate more number of serum samples in a given time to assess the flock immunity, single serum dilution ELISA was preferred (Snyder *et al*., 1983). This method saves large scale cost on reagents and time, when compared to other serological tests. The ELISA plates can be coated with antigen in advance and stored even for one year period. When precoated plates are used the test can be done in less than 5 hours.

Moreover, in ELISA, the antigen-antibody complex and antibody-conjugate complexes were subjected to rigorous washes to eliminate excess reagents at each incubation step. This process tends to favour the binding of only high affinity and avidity antibodies. So, even in lower concentrations also, the antibodies bind to the antigen. In single serum dilution method, the data was subjected to regression analysis, which has a major advantage in that there is a little scope for discrepancies if any. Hence, the single serum dilution method could be used as a reliable method for detection of IBDV antibodies. However, the developed single serum dilution ELISA could not be compared to VNT (gold standard test) during the present study. Hence, further studies would further validate the single serum dilution ELISA. Further, the assay must be subjected for both intra and inter laboratory validation. Several studies reported the use of FTA for the detection of hormones, insecticides, serum antibodies and viral antigens (Wang *et al.*, 2005; Venkatesh, 2006). In the present study, the FTA was able to detect 200 ng of purified viral antigen. While standardizing the assay, a range of purified IBDV antigen concentrations were used viz. 50, 100, 150, 200 and 250 ng/µl. A strong positive test as evidenced by a clear dot development was observed with an antigen concentration of 100 ng / µl onwards. However, the dot was clear and optimum in 200 ng/µl concentration. After the initial coating of antigen to the cellulose acetate membrane the reaction time between the antigen and antibody was very short. Hence, only the high affinity antibodies present in the hyperimmune serum could bind to the antigen strongly and color development in the form of dot was noticed immediately on the addition of conjugate. During the assay development nitrocellulose membrane (0.22µm) was initially used. But, due to the high background following addition of colloidal gold conjugate, it was subsequently replaced by cellulose acetate membrane which gave good result with very little background. The protein - A part in the conjugate binds to the Fc portion of mammalian IgG naturally. The positive control in FTA was rabbit serum instead of antigen. The conjugate binds to the Fc portion of the inmmunoglobulin, while the high affinity antibodies only bound to coated antigen on membrane.

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