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Development of single serum dilution ELISA for detection of infectious bursal disease virus antibodies

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ABSTRACT

Determination of antibody titres of sera collected from poultry flock to diagnose IBDV infected chickens by developing single serum working dilution ELISA (SSD-ELISA) was made. The indirect ELISA standard procedure was adopted with standard positive and negative serum controls. The standard IBDV antigen was prepared using the Tamil Nadu isolate of IBDV. The relative sensitivity and specificity of a single serum dilution ELISA test in qualitative comparison with the commercial kit were 82% and 100% respectively and the accuracy was 82.1%. This single serum dilution assay gave reproducible results and allowed considerable savings on the time and cost of reagents compared with indirect ELISA. Based on these results a SDS-ELISA kits have been developed in this study to replace the commercial kit. The IBDV antigen coated ELISA plates can be stored under refrigeration and the test can be performed rapidly under field conditions by trained personnel.

Key words: serum, ELISA, infectious bursal disease, antibody titer

Introduction

Infectious bursal disease outbreaks have been reported in India since 1971 (MONANTY et al., 1971) and in Tamil Nadu since 1988 (PURUSHOTHAMAN et al., 1988). Outbreaks have occurred in different areas of Tamil Nadu since 1973. The incidence of infectious bursal disease was first reported in Northern India and the virus was isolated and identified in 1974 (JAYARAMAIAH and MALLICK, 1974). The prevalence of hypervirulent IBD virus in different areas of Tamil Nadu, India was confirmed by RAMADASS et al. (2003).

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The serological survey of infectious bursal disease virus specific antibodies was done by employing the immunodiffusion test. Quantitation of antibodies to infectious bursal disease was studied by CULLEN and WYETH (1975). Serological study of antibodies was carried out by imunocomb based dot-enzyme linked immunosorbent assay (MANOHARAN et al., 2004). AMIYA et al. (1991) developed an indirect ELISA for measuring antibodies against IBD for the first time in India.

In single-serum-dilution ELISA, the antibody titer of a serum sample is predicted from the absorbance value obtained in a single dilution of serum. This technique reduces cost and saves the time of the routine indirect ELISA. This technique is significant because of the near linear relationship between the antibody titer and absorbance value at single working dilution (SNYDER et al., 1983). SNYDER et al. (1984) standardized single dilution ELISA for IBD by the same method used for Newcastle Disease. SOLANO et al. (1985) used the kinetics of enzyme substrate reaction to evaluate a single serum dilution, indirect ELISA for quantification of IBDV antibodies in White Leghorn hens inoculated with infectious bursal disease vaccines. The linear relationship between titre and colour reaction (slope) was directly proportionate to the quantity of the reaction curve, the higher the titre of the antibody. BRIGGS et al. (1986) used positive/negative (P/N) ratio method of analysis to quantitate antibody response to IBDV. In this study an indigenous SSD-ELISA kit was developed and the sensitivity and specificity were compared with the commercial kit.

Materials and methods

Virus. The infectious bursal disease virus isolated from Tamil Nadu was used in this study. This isolate was propagated in Vero cell culture and purified using 30% and 60% sucrose gradient centrifugation. The partially purified IBD (TN) virus was used as the antigen for coating ELISA plates.

Purification of antigen. The antigen of IBDV was purified using the methods described by SNYDER et al. (1983). Infected Vero culture supernatant was clarified at 10,000 rpm for 20 min at 4 ^{0}C in a cooling centrifuge (Sigma, Germany) to remove the cell debris. The clear supernantant was pelleted by ultracentrifugation at 35,000 rpm for 3.30 hrs, at 4 ^{0}C (Beckman Ti 70 rotor, USA). The viral pellet was resuspended in one-tenth of the original volume in TNE buffer (0.01 mol/L Tris HCl, 0.1 mol/L NaCl and 0.001 mol/L EDTA, pH 8.3). The suspension was centrifuged again at 10,000 rpm for 10 min at 4 ^{0}C to remove cell debris. The supernantant was collected and loaded onto 30% and 60% discontinuous sucrose gradient. The gradient was then centrifuged at 35,000 rpm for 3.30 has at 4 ^{0}C (Beckman Ti 60 rotor). After centrifugation, the band at the interface of the 30% and 60% sucrose layers were collected and diluted approximately 1:5 in TNE buffer, and stored at -70 ^{0}C and protein concentration was quantified as 7 mg/mL

Indirect ELISA procedure. Antigen was diluted at 1:75 in carbonate and bicarbonate buffer pH 9.6 and 100 µL of diluted virus was added to each well. The plate was incubated at 4 ^{0}C overnight for antigen coating. The wells were washed three times with PBST. Antigen coated wells were blocked using 2% BSA. One hundred µL blocking solution (PBST-BSA) was added to each well, incubated at 37 ^{0}C for 1 hr and washed three times with PBST. Serum samples were diluted to 1:100 in PBS and two fold dilutions were made serially up to 1:12800. From this dilution, 100 µL was added in duplicate to antigen coated wells. The plate was then incubated at 37 ^{0}C for 1 hr, washed three times with PBST and added 100 µL of 1:3000 dilution of rabbit-chicken IgG HRP conjugate in PBS. After 1 hr incubation at 30 ^{0}C, the wells were washed three times with PBST and 100 µL of freshly prepared substrate solution (ABTS with H_2O_2) was added and incubated for 5 min at 37 ^{0}C. The reaction was stopped by adding 50 µL of 5% SDS solution and the absorbance was measured at a wavelength of 405 nm in the ELISA reader.

Indirect ELISA was run with the hyperimmune serum raised in rabbits against IBDV as a positive serum control and serum of unvaccinated birds as a negative serum control. Air blank, serum control, conjugate control and substrate control was also included. To decrease the variation between different plates, the titre of sera were estimated using the sample to positive ratio (S/P ratio) calculated as follows.

$$\text{S/P ratio} = \frac{\text{Sample OD - Negative OD}}{\text{Positive OD - Negative OD}}$$

Positive/negative threshold baseline (PNT baseline)

The PNT baseline was constructed as per the method of SNYDER et al. (1983). The PNT baseline was used to determine the endpoint in serial dilution ELISA. A total of 12 different negative serum samples were subjected to indirect ELISA separately. Mean absorbance plus three standard deviation units were calculated at each dilution for these 12 negative serum samples. The resulting three standard deviation baseline unit values were then plotted against each dilution on a graph sheet in Microsoft Excel and the PNT baseline was used for calculation of the observed antibody titres.

Corrected average absorbance. Average absorbance of test serum samples were corrected by substracting the appropriate average absorbance of internal negative control serum dilutions from the same trial (SNYDER et al., 1983).

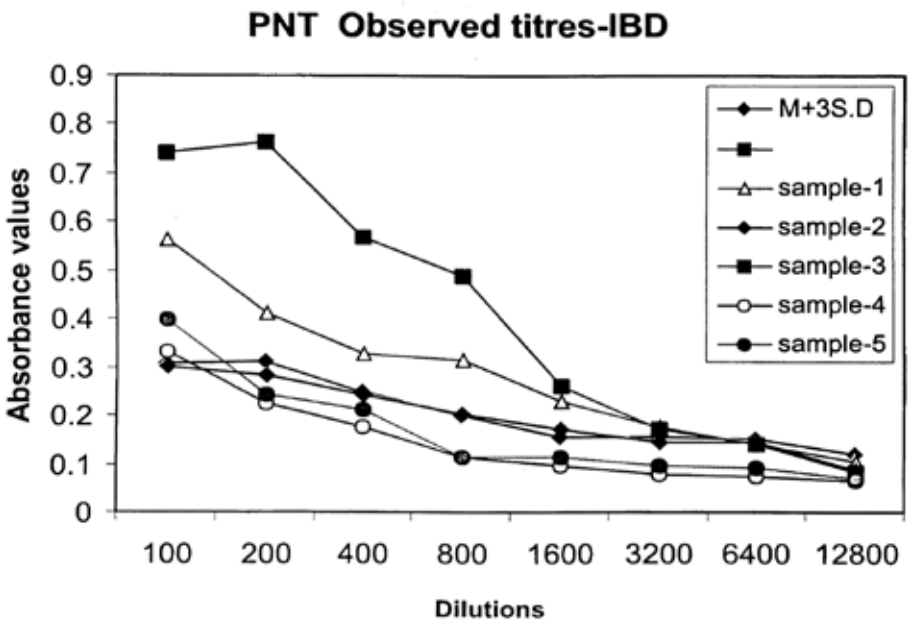

Fig. 1. Standard serial dilution method of determining observed ELISA antibody titres with PNT baseline. M+3 SD indicates positive and negative threshold (PNT) baseline.

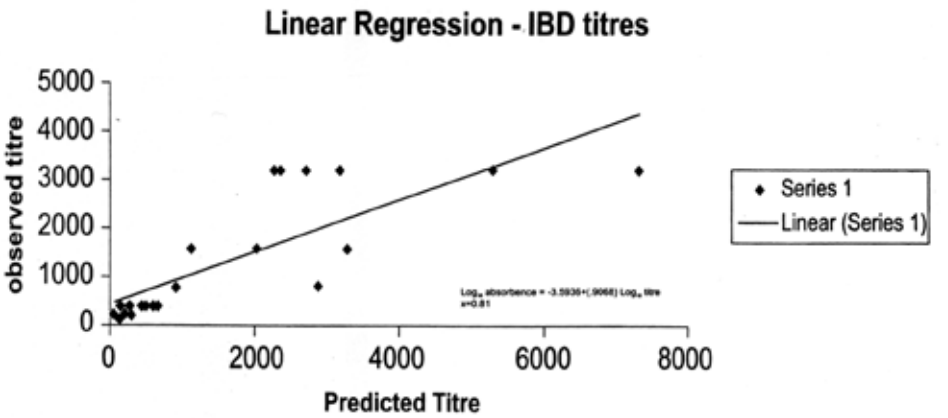

Fig. 2. Relationship between IBD antibody titres of 44 field sera in ELISA and their predicted titres of 1:800 dilution. Correlation coefficient and regression equation are shown.

Observed ELISA antibody titer. The corrected average absorbance of test samples were graphed and the observed antibody endpoint titer was defined as the point at which the plotted lines intersect the PNT base line (Fig.1.) (SNYDER et al., 1983).

Standardization of Single serum dilution ELISA. Random sera samples of about 44 birds were subjected to indirect ELISA and observed antibody titer for the samples were determined as in the method described above. The regression analysis was carried out with the $\log_{10}$ values of the corrected average absorbance at 1:100 and 1:800 of each serum samples with their corresponding $\log_{10}$ observed antibody titres. The dilution 1:800 at which highest correlation (0.81) obtained would be taken as a single working dilution to predict the antibody titre. A linear regression line and a standard regression equation were obtained at this dilution (Fig. 2). Regression analysis was done using Microstat Statistics software package. The regression equation was generated by comparing the $\log_{10}$ S/P ratio of samples tested at 1:800 dilution with their corresponding observed titres as follows.

Log_{10} (S/P ratio) = Intercept + Slope x $\log_{10}$ titre or rewritten as

Log_{10}titre = (1/Slope x $\log_{10}$ (S/P ratio) - (intercept slope)

Predicted antibody titer. The corrected absorbance at single working dilution (1:800) of serum samples was directly converted into antibody titer (predicted antibody titer using the standard regression equation). Subsequent samples were tested in duplicate at this dilution, the $\log_{10}$ S/P ration was calculated for each sample and using the regression equation developed their predicted titres were calculated. Positive and negative controls were included in every plate as controls. When the mean absorbance for the negative control exceeded 0.2, the results were rejected.

Statistical analysis. A linear relationship existed between the observed antibody titres and the predicted antibody titres of the 44 samples at 1:800 working dilution using the regression line equation. The relationship between observed and predicted antibody titres is highly significant. The sensitivity, specificity and accuracy were calculated by comparing the developed ELISA with commercial ELISA kit (IDEXX laboratories, USA).

Results

The mean plus three standard deviation (M+3 SD) values of the 12 different negative sera samples at serial dilutions from 1:200 were 0.306, 0.310, 0.248, 0.199, 0.156, 0.155, 0.149 and 0.119. Using these values as cut off, the PNT line was determined. The observed titre was determined as the dilution at which the sample absorbance crosses the PNT line. The dilution of the serum where the S/P ratio best reflect the different titres as determined by serial dilutions was found to be 1:800 based on the highest correlation

coefficient obtained (Table 1). Based on these values, the regression equation formulated was $0.0958 \times (\log_{10} S/P \text{ ratio}) + 3.5936$). Thus the serum samples were tested in duplicate at a single dilution of 1:800, the $\log_{10}$ S/P ratio determined and the ELISA titers predicted.

Table 1. Constants of the regression line for the 3 selected dilutions of serum tested in ELISA

Serum Dilution	Correlation coefficient	Intercept (C)	Slope (m)
1:200	0.4591	- 0.9691	0.41078
1:400	0.7390	- 2.4061	0.62097
1:800	0.810	- 3.5936	0.90685

Table 2. Relative sensitivity, specificity and accuracy of the developed ELISA to detect IBD virus specific antibodies using the commercial kit as a reference standard

Technique	Commercial kit positive	Commercial kit negative	Total
Developed ELISA positive	116 (a)	0 (b)	116
Developed ELISA negative	25 (c)	7 (d)	32
Total	141	7	148

Sensitivity: a/a+c = 82.0%; Specificity: d/b+d = 100%; Accuracy: a+d/a+b+b+d = 83.1%

For 44 samples tested initially the difference between the predicted ELISA titres and the actually observed titres were never greater than one two fold dilution. The correlation coefficient of the developed ELISA with commercial ELISA kit as the gold standard are shown in Table 2. This was a qualitative comparison using commercial ELISA kit titres greater than 396 as positive and a predicted ELISA titre value of >900 as positive. The correlation coefficient of the developed ELISA and the commercial ELISA kit was 0.820.

Discussion

The method and technique described here is directed toward implementing ELISA as a reproducible and practical clinical tool for resolving the immune status of a population so that early management decisions regarding vaccination can be made.

This also affords economy so that the sample size can be increased since by using the two fold serial dilution method only about 6 - 7 samples can be evaluated in a single ELISA plate using dilutions from 1:100 to 1:12800. However, by using the developed

single serum dilution ELISA method, a maximum of 44 samples can be analyzed and their antibody titres quantitated with sufficient accuracy.

Using the developed regression equation and testing the serum at a single dilution of 1:800, the predicated titre were close to the actually observed titres arrived using serial dilutions of serum and their correlation coefficient was 0.81. The relative sensitivity and specificity of a single serum dilution ELISA test in qualitative comparison with the commercial kit were 82% and 100% respectively and the accuracy was 82.1%. The equation in the sensitivity may be attributable to the more false negatives shown by the developed ELISA. But all the false negatives by developed ELISA were confirmed negative by AGID also.

References

AMIYA, K. A., A. RAO., N. K. PARTHUI (1991): An indirect ELISA for detection of antibodies in chicks infected with infectious bursal disease virus. Indian Vet. J. 68, 1109-1112.

BRIGGS, D. J., C. E. WHITFILL, J. K. SKEELES, J. D. STORY, K. D. REED (1986): Application of the positive/negative ratio method of analysis to quantitate antibody responses to infectious bursal disease virus using a commercially available ELISA. Avian Dis. 30, 213-218.

CULLEN, G. A., P. J. WYETH (1975): Quantitation of antibodies of infectious bursal disease. Vet. Rec. 97, 315.

JAYARAMAIAH, B., B. B. MALLICK (1974): Identification of infectious bursal disease virus in India. Ind. J. Anim. Hlth. 13, 99-2103.

JAYARAMAIAH, B., B. B. MALLICK (1975): Serological survey of infectious bursal disease. Ind. Vet. J. 52, 673-676.

MOHANTY, G. C., A. P. PANDA, B. S. RAJYA (1971): Infectious bursal disease in chicken. Curr. Sci. 40, 181-184.

MANOHARAN, S., M. PARTHIBAN, T. G. PRABHAKAR, G. RAVIKUMAR, A. KOREESWARAN, N. D. J. CHANDRAN, G. RAJAVELU (2004): Rapid serological profiling by an immunocomb based dot-enzyme linked immunosorbent test for three major poultry diseases. Vet. Res. Commun. 28, 339-346.

PURUSHOTHAMAN, V., S. P. PANNEERSELVAM, K. VENUGOPAL, T. M. THANGARA (1988): A report on the outbreak of infectious bursal disease in chickens. Cheiron 17, 229-231.

RAMADASS, P., V. THIAGARAJAN, M. PARTHIBAN, T. M. A. SENTHIL KUMAR, D. LATHA, A. ANBALAGAN, M. KRISHNAKUMAR, K. NACHIMUTHU (2003): Sequence analysis of infectious bursal disease virus isolates from India: phylogenetic relationships. Acta virol. 4, 131-135.

SOLANO, W., J. J. GIAMBRONE, V. S. PANANGALA (1985): Comparison of a kinetic enzyme linked immunosorbent assay (KELISA) and virus neutralization test IBDV. I. Quantitation of antibody in white leghorn hens. Avian Dis. 29, 662-671.

SNYDER, D. B., W. W. MARQUARDT, E. T. MALLINSON, E. RUSSEK (1983): Rapid serological profiling by enzyme linked immunosorbent assay. I. Measurement of antibody activity titer against Newcastle disease virus in a single serum dilution. Avian Dis. 27, 161-170.

SNYDER, D. B., W. W. MARQUARDT, E. T. MALLINSON, P. K. SAVAGE, D. C. ALLEN (1984): Rapid serological profiling by enzyme linked immunosorbent assay. III. Simultaneous measurements of antibody titers to infectious bronchitis, infectious bursal disease and Newcastle disease viruses in a single serum dilution. Avian Dis. 28, 12-24.

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RAMADASS, P., M. PARTHIBAN, V. THIAGARAJAN, M. CHANDRASEKAR, M. VIDHYA, G. D. RAJ: Razvitak imunoenzimnog testa s jedostrukim razrjeđenjem seruma za dokaz protutijela za virus zarazne bolesti burze. Vet. arhiv 78, 23-30, 2008.

SAŽETAK

Titar protutijela za virus zarazne bolesti burze određen je u uzorcima seruma pilića razvijenim imunoenzimnim testom s jednostrukim razrjeđenjem uzoraka seruma. Standardni imunoenzimni test prilagođen je uz uporabu standardnih pozitivnih i negativnih kontrolnih uzoraka seruma. Standardni antigen za virus zarazne bolesti burze bio je pripravljen od izolata Tamil Nadu virusa. U usporedbi s komercijalnim kompletom, osjetljivost testa iznosila je 82%, a specifičnost 100%. Točnost mu je bila 82,1%. Razvijenim testom postignuti su reproducibilni rezultati uz znatnu uštedu vremena i materijala pa se može uporabiti umjesto komercijalnih kompleta. Antigen se može pohraniti u hladnjaku, a uvježbano osoblje može brzo izvesti test u terenskim uvjetima.

Ključne riječi: zarazna bolest burze, imunoenzimni test, serum, titar protutijela