J. Vet. Anim. Sci. (2008), Vol. 1: 18-23

Standardization of an Indirect Enzyme Linked Immuno Sorbent Assay for Measuring Antibodies of Infectious Bursal Disease Virus

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ABSTRACT

An attempt was made to standardize an in-house immuno-enzyme assay for measuring antibodies of infectious bursal disease virus (IBDV) in chicken. The test was performed after coating plates with ELISA antigens prepared by two methods. Antigen "A" was prepared from infectious bursal disease virus infected chicken embryo fibroblasts culture by concentration with dialysis against PEG-6000 while antigen "B" was prepared reconstituting a live infectious bursal disease virus vaccine. The optimum dilution of antigen "A" and "B" was found to be 1:300 and 1:600 respectively. Both the antigens produced acceptable and comparable results but antigen "B" is conventional due to ease of preparation and to avoid a time consuming and costly procedure of cell culture. The rabbit anti-chicken immunoglobulin-G conjugated to horseradish peroxidase was used at a dilution of 1:2000. The assay was evaluated by testing chicken serum samples of different age groups (1-day-old broiler breeder and broiler chicks, 13 weeks old vaccinated layer breeder birds and 30 week old vaccinated broiler breeder birds). The efficiency of the standardized ELISA was compared with a commercially available ELISA kit. The results indicated that in-house developed ELISA was equally as sensitive and specific as commercially available kit in detection of antibodies against IBDV.

***Key Words*:** Enzyme linked immuno sorbent assay, Infectious bursal disease

INTRODUCTION

Infectious bursal disease (IBD) caused by a Birnavirus was first reported in Pakistan by Khan et al. (1988) and now it has become a major poultry disease during the past 5-7 years in layers and broilers (Ahmed and Akhter, 2003). The disease has shown mortality, sometimes as high as 23% in a few isolated outbreaks (Anjum et al., 1994). When the chickens are infected at an early age, they display a severe and prolonged immunosuppression, compromising both humoral and cellular responses of chickens (Lukert and Saif, 2003). Immunization of chickens is a principal method used to control IBD. Immunization of breeder flocks confers the maternal immunity to their progeny. The most commonly used methods to detect antibodies of infectious bursal disease virus include agar gel precipitation test (AGPT), virus neutralization (VN) and enzyme linked immuno sorbent assay (ELISA) (Weisman and Hitchner, 1978). The AGP test is an economical and simple to perform. However, it is not much sensitive as it fails to detect antibodies in birds even they are resistant to field challenge. The VN test appears to be a better indicator of flock immunity, but it is more expensive, cumbersome and time consuming.

In contrast, ELISA is the most commonly used serological test for the evaluation of IBDV antibodies, as it is sensitive, precise

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and rapid to perform. The cost of imported ELISA kit is usually very high and not affordable by the average farmers. The present study was designed with an aim to develop and standardize a cost effective ELISA kit for detection of IBD antibodies in chicken.

MATERIALS AND METODS

Propagation of Infectious Bursal Disease Virus in Cell Culture

The infectious bursal disease virus was propagated in primary chicken embryo fibroblast (CEF) cells. CEF were prepared from 9-11-days-old embryonated chicken eggs. The eggs were collected from the flock of layers reared in the Department of Microbiology, University of Veterinary and Animal Sciences, Lahore. Cell culture medium M-199 (ICN Biomedicals, Inc., Costa Mesa, Calif) was used to culture CEF. Antibiotic solution was added @ 100 IU of penicillin, 100 μg of dihydrostreptomycin sulfate and 0.25 μg amphotericin-B per milliliter of medium.

The CEF were prepared as described by Karel and Purchase (1998) with minor modification. A group of 9-11 days-old embryonated eggs were candled for confirming the viability. Viable embryonated eggs (9-11 days-old) were cracked on the air space end and embryos were removed. Embryos were placed in glass petri plate containing cell culture medium (CCM) without serum for washing. Embryos were eviscerated along with the removal of head and limbs. The remaining tissues were washed again and finely minced. The finely minced tissue was transferred to a trypsinization flask containing trypsin and antibiotic solution. The tissues were stirred with magnetic bar for twenty minutes at room temperature. After trypsinization the cell suspension was passed through a sterile muslin cloth. The filterate thus collected was centrifuged at 1000 rpm for 4 minute. The supernatant was poured off and the cell pellet was re-suspended in cell culture medium M-199 supplemented with 10% calf serum. (ICN Biomedicals, inc., Costa Mesa, Calif.

A commercially available live IBD vaccine D-78 (Intervet Inc, Millsboro, DE), 1000 doses was re-suspended in 5mL of sterilized normal saline. This inoculum was used to infect CEF cells. At 80% cell confluency of the fibroblasts CCM was removed. An aliquot of 0.5 mL of re-suspended live IBD virus vaccine was added to each $25cm^2$ culture flask. The virus was allowed to adsorb on the cells for some time at 37°C. The excessive virus was removed and maintenance medium supplemented with 2% calf serum was added. The flasks were incubated at 37°C. The cells were observed for the development of cytopathic effects (CPE) regularly after every 24 hours post infection for 3-4 days.

Upon appearance of generalized CPE, the flasks were removed from the incubator and placed in a freezer. The CEF monolayer was disrupted by three freeze-thaw cycles and the suspension was centrifuged at 8,000 rpm for twenty minutes at 4°C. The supernatant was collected and stored at -70°C in small aliquots till further use.

***Agar Gel Precipitation Test*:** AGPT was performed as described earlier (Anonymous, 2004) with minor modification.

Source of IBDV Reference Antisera

***Rabbit Antisera*:** The rabbit (n = 2) injected with an oil adjuvanted vaccine of IBDV (Merial Animal Health, CM), @ 0.5 mL/rabbit subcutaneously on 0, 10^{th}, 20^{th} and 30^{th} day. The blood was collected prior to vaccination and at 20^{th} and 40^{th} days post vaccination. The collected sera were checked for the presence of IBDV antibodies by AGPT against reference IBD virus D-78. The sera found positive were used as positive control serum.

One of the rabbit was kept as negative control and was not vaccinated. The blood was collected at 40th day and serum was screened for the IBDV antibodies in AGPT.

***Chicken Antisera*:** Five cockerels of two months age were vaccinated with live and oil adjuvanted vaccine of IBDV through eye dropping and subcut routes at 0, 14th and 28thdays. The blood was collected prior to vaccination and at 14th, 28th and 42nd days post vaccination. The collected sera were checked for the presence of IBDV antibodies by AGPT against reference IBD antigen. The sera found positive through AGPT were pooled to use as positive control IBDV reference antisera for further use.

For negative control sera twenty broiler birds were raised in an isolated place. The birds were not vaccinated with any IBD vaccine. Blood was collected after 40 days. The sera collected were pooled and checked by AGPT to confirm the absence of IBDV antibodies.

Preparation of ELISA Antigen

IBDV antigen required for coating polystyrene micro plates was prepared with two different techniques to perform indirect ELISA. These were as follows

***Antigen "A"*:** It was prepared from IBDV propagated on primary CEF as described earlier.

***Antigen "B"*:** A live IBDV vaccine was also evaluated to be used as antigen (Ahmed et al., 2003).

***Standardization of Indirect ELISA*:** An indirect ELISA was developed for the detection of antibodies against IBDV in chicken sera. The assay was standardized using two antigens against the known positive and negative chicken sera raised in experimental birds.

***Standardization of ELISA Antigens*:** The optimum dilution of the antigen required for coating ELISA plates was determined. Two-fold serial dilution of the antigen was made in carbonate/bicarbonate coating buffer pH 9.6. The plates were covered and incubated in an airtight humid box at 4°C for 18 hours. The plates were washed thrice with TEN-T (Tris 60.5g, EDTA 3.7g, NaCl 87.7g, Tween20 4.4g per liter, pH 8.0) buffer. Positive and negative control sera were diluted 1:100 containing TEN-TC also (Tris 60.55g, EDTA 3.7g, NaCl 87.70g, Tween 20 4.4g, casein 20 g/L, pH 8.0). Thus four replicates of each dilution were tested against positive control serum as well as negative control serum. The plate was incubated for half hour at room temperature and washed thrice with TEN-T. The anti-chicken conjugate was diluted @ 1:1500 and an aliquot of 100 µL/well was added in all the wells. . The plate was again incubated for half hour at room temperature and washed thrice with TEN-T. After washing an aliquot of 100 µL/well ABTS substrate were added. The plate was read at 405 nM wavelength after 15, 30, 45 and 60 minute interval. The highest dilution of antigen producing maximum contrast between positive and negative control sera was used for the assay.

***Standardization of Conjugate*:** The rabbit anti-chicken IgG whole molecule conjugated with horseradish peroxidase (ICN Biomedicals, Inc., Costa Mesa, Calif) was standardized to perform ELISA. The working dilution for the each batch of conjugate was determined by diluting it in dilution buffer (TEN-TC) @ 1:1500, 1:2000 and 1:2500 against positive and negative control sera after coating appropriate antigen dilutions of both antigens A and B. The assay was performed to select the standard conjugate dilution producing maximum contrast between positive and negative control sera with the least deviation from the mean.

***Enzyme substrate solution*:** The substrate solution was prepared in a citrate/phosphate buffer (pH 4.2) by mixing 2-2 azino-di-3 ethyl-benzthiazoline sulphonic acid (ABTS) 52mM (0.2mL), Hydrogen per oxide 130mM

(0.2mL) and citrate/phosphate buffer 0.1 M (10mL). The substrate solution was added in 100µL quantity per well and plates were incubated in dark. Readings were taken after 15, 30, 45 and 60 minute at wavelength of 405 nm.

***Standard Curve*:** The standard curve was constructed using serial two-fold dilution of hyperimmune chicken serum in diluent buffer (TEN-TC) (Figure 1).

***Evaluation of Developed ELISA*:** The standardized in-house ELISA was evaluated using the sera collected from various poultry farms. A total of 38 serum samples with different vaccination histories were tested. Samples were divided in to four groups namely A, B, C and D. Groups A and B comprised the serum samples of a day-old chicks from broiler breeder and broiler flocks, respectively. Group C comprises the serum samples of 13 weeks old vaccinated layer breeder birds. Serum samples of 30 weeks old vaccinated broiler breeder birds were assigned group D. All samples were diluted 1:100 in TEN-TC for use in indirect ELISA. An aliquot of 100µL/well were added in antigen-coated plates. The results obtained from in house ELISA were compared with a commercial ELISA kit (IDEXX Laboratories, Inc. Westbrook, Maine) (Table 1).

Table 1 Comparison between Mean O.D (Means +-SD) values of different groups tested with in-house ELISA and commercial ELISA kit

Groups	Optical density		
	Antigen "A"	Antigen "B"	Kit
A (n = 8)	1.022 ± 0.574	0.933 ± 0.661	0.733 ± 0.207
B (n = 10)	0.158 ± 0.101	0.134 ± 0.47	0.207 ± 0.126
C (n = 10)	1.999 ± 0.347	1.23 ± 0.255	0.64 ± 0.156
D (n = 10)	2.639 ± 0.357	2.113 ± 0.63	0.797 ± 0.213

RESULTS AND DISCUSSION

In the present study chicken embryo fibroblast (CEF) cell culture was used to propagate the IBDV. The monolayer of CEF cells was ready after 24–36 hours as documented by early workers (Karel and Purchase, 1998; Srivastava et al., 2001). The CPE were observed in virus-infected cells after 36 hours. The CPE were characterized by rounding and granulation of cells around the nucleus. Subsequently detachment of cells from the flask surface with complete destruction occurred after 72 hours. Similar time frame of events was observed by Srivastava et al. 2001. The propagation of IBDV in infected cell culture supernatant was confirmed by AGPT against specific IBDV antisera raised in rabbit and chicken. The IBDV harvested after every passage showed strong precipitation reaction with reference to rabbit and chicken IBDV anti sera after 24 hours of incubation. The sera of vaccinated rabbits and chickens prior to vaccination were found negative while it showed positive reaction at least after second shot of vaccination. The negative control sera did not show any precipitation line up till 96 hours of incubation. It was recorded as 1:300 for antigen "A" and 1:600 for antigen "B" respectively. Both the antigens produced acceptable and comparable results. However antigen "B" is recommended, as it is ready to use and to avoid a time consuming and costly procedure of cell culture. The incubation time for substrate was

standardized at room temperature (27-30°C). The standard curve (for both antigens 'A' and 'B' was constructed to record the optical densities (O.Ds) produced by the serial two fold dilution of the control positive IBDV antisera in TEN-TC. Eleven dilutions were used to perform the assay and a graph of O.D values was plotted. Figure 1 represents the O.D values of eleven dilutions of positive control chicken IBDV antisera with antigens A and B. Three out of these eleven dilutions were selected and designated as standard 1, 2 and 3. The mean absorbance of 1st selected standard (dilution no. 7) using antigen **'A'** was recorded 0.51 and with antigen B it produced the O.D value of 0.425. The O.D value of test sera above standard 1 was considered strong positive. Mean absorbance values of second standard (dilution no. 8) on antigen 'A' and 'B' were recorded as 0.321 and 0.226 respectively. The standard 2 was selected as the cut off value between positive and negative. The O.D values falling between standard 1 and 2 were considered moderately positive. The third selected standard (dilution no. 11) had a mean O.D value of 0.139 and 0.196 on antigens 'A' and 'B' respectively. Any O.D value equal or below standard 3 was considered negative. Poultry field serum samples of different age groups were used to evaluate the in-house ELISA and its efficiency was compared with a commercially available ELISA kit. Group A comprises the 1-day-old chicks of broiler breeder flock. The mean O.D values of different groups A, B, C and D are represented in Table 1. The absorbance values of maternally derived antibodies of group A were above standard No. 1, as detected by both in-house and commercial ELISA. The serum samples of this group were considered strong positive. Serum samples of group B (1-day-old broiler chicks) showed O.D values below standard 3 with in-house and commercial ELISA. All samples of group B were negative for antibodies against IBDV. The antibodies in 1-day-old chicks varied from flock to flock and depended upon the maternal antibody titer of the parent flock. Normally titers in progeny were 60-80 % lower than those in the parent (Lukert and Saif, 2003).

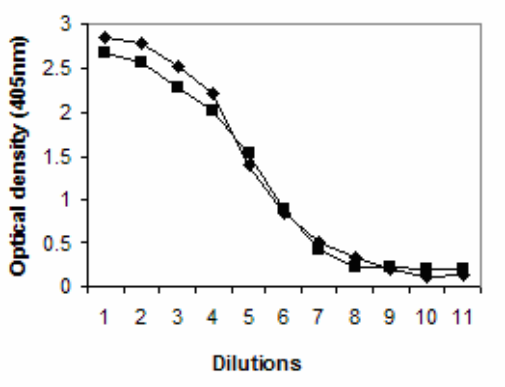

Figure 1 Optical density of positive control chicken IBDV antisera with antigen "A" and "B"

Group C (13-week layer breeder birds) of age. O.D values of serum samples of this group were also above standard no. 1 (i.e. strong positive). It showed clearly that vaccination had produced a high level of antibodies, detectable by both in-house and commercial ELISA. Group D (30 weeks old broiler breeder) were vaccinated 4 times at 8th, 18th and 133rd day of age with live and killed vaccines. The serum samples of this group also showed a high level of antibodies as a result of immunization. The absorbance values were above standard No. 1 even higher than that of group C that was vaccinated thrice. Generally it was noted that O.D values of test serum samples were higher with the antigen "A". The antigen "B" showed O.D values a little lower than antigen "A" while the O.D values with commercial ELISA kit was the lowest.

There might be certain factors for this pattern; one factor might be the dilution of test serum samples. The dilution used for in-house ELISA was lower as compare to commercial kit. The lower dilution for in-house ELISA was selected to increase the

sensitivity of the test, as the virus used as an antigen for in-house ELISA was not purified. Second factor for higher absorbance value of in-house ELISA might be the presence of some non-specific unidentified proteins in the antigen. The higher O.D values suggested that higher dilutions of sera could be used. In spite of the dilution factor, it was observed that almost all the samples that were found positive or negative with commercial ELISA kit also showed the similar pattern with in-house ELISA. The pattern of increasing or decreasing O.D values was quite similar.

It was concluded that in-house developed ELISA with both antigens A and B compared well with commercial ELISA kit.

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