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Evaluation of Competitive Enzyme Linked Immunosorbant Assay for Detection of Antibodies to Bovine Herpes Virus -1

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ABSTRACT

The present study was carried out to make a comparative evaluation of a commercial competitive ELISA kit with respect to gold standard micro-serum neutralization test (mSNT) for detection of antibodies to Bovine herpes virus -1. The relative sensitivity and specificity of cELISA were found to be 96.67% and 95.29%, respectively. The positive and negative predictive values were observed to be 78.38 and 95.29%, respectively. The exhibited *kappa* value was 0.95. From this study, it is evident that cELISA is very simple, rapid with high specificity and sensitivity in detecting BHV-1 antibodies and can prove to be a quick and effective tool in diagnosis of the disease in field conditions where mSNT cannot be carried out due to its inherent requirement of highly specialized laboratories with cell culture facilities and expert personnel.

Keywords: cELISA, mSNT, antibodies, BHV-1

Bovine herpes virus 1 (BHV-1) infection in bovines is an economically important viral disease that occurs throughout the world including India (Verma *et al.,* 2014; Thakur *et al.,* 2015). The BHV-1 can be differentiated into subtypes BHV-1.1, BHV-1.2a and BHV-1.2b on the basis of restriction enzyme profiling of genomic DNA (Metzler *et al.,* 1985). Subtype 1.1 is mainly responsible for the respiratory form of the disease whereas 1.2a and 1.2b mainly affect the reproductive system. However, BHV-1.1 may also be associated with the reproductive form of the disease. Bovine herpesvirus 1.2 subtypes may be less virulent than subtype 1.1. Bovine herpesvirus subtype 1.2a is abortigenic whereas 1.2b is not abortigenic (Edwards *et al.,* 1990).

BHV-1 infection is highly infectious viral disease of bovines characterized by mild to severe respiratory or genitourinary diseases such as infectious bovine rhinotracheitis (IBR), infectious pustular vulvovaginits, infectious pustular balanoposthitis (IPB), conjunctivitis, encephalitis and abortion The virus is also associated with enteritis, loss of milk production, loss of draught power, infertility and immunosuppression (Gibbs and Rweyemamu, 1977; Miller, 1991; Saravanajayam *et al.,* 2015).

The disease was first reported in India by Mehrotra *et al.* (1976) and thereafter has been reported from various parts of India in the bovine population by various workers (Renukaradhya *et al.,* 1996; Nandi *et al.,* 2011; Kollanur *et al.,* 2014; Verma *et al.,* 2014; Thakur *et al.,* 2015).

Preliminary diagnosis of the disease can be made by its clinical manifestations, but for confirmatory diagnosis the virus isolation in cell culture, fluorescent antibody technique, ELISA, virus neutralization test (VNT), and PCR are commonly used (OIE, 2008; Nandi *et al.,* 2009). The virus neutralisation test (VNT) and enzyme-linked

immunosorbent assay (ELISA) are usually employed to detect the acute as well as convalescent stage of infection. These tests can also detect the latent or carrier stage and have important implications for international trade as well as epidemiological studies (Guarino *et al.*, 2008; OIE, 2004).

The identification of infected animals require sensitive and reliable serological tests at field level which can aid in quick detection of infected animals and following their segregation from the herd will prevent the transmission of the virus, thereby helping in implementation of successful control programme. Various workers have suggested different ELISA as reliable tests for routine detection of BHV-1 antibodies instead of SNT for rapid detection of infected animals so as to prevent the non infected animals from infection (Cho and Bohac, 1985; Kramps *et al*., 1994; Neves *et al.*, 2009).

The mSNT is considered to be the 'gold standard' and an OIE recommended test (OIE, 2008). Though mSNT is considered to be the best of all serological assays, there is a need of an assay which has sensitivity and specificity closer to mSNT and is relatively easy and rapid to perform in the Indian field condition. Keeping this in view samples were assayed using mSNT and cELISA and relative sensitivity as well as specificity of cELISA were also calculated considering mSNT as gold standard test.

MATERIALS AND METHODS

Sample collection

A total of 200 blood samples collected in clot activator containing serum vacutainer from bovines of different farms of Uttarakhand, India. Serum was separated; centrifuged anderum was stored at -20ºC in a sterile screw caped vial for further analysis.

The serum samples were tested both by mSNT and cELISA at Virology laboratory, CADRAD, Indian Veterinary Research Institue, Izatnagar, Bareilly, Uttar Pradesh, India to detect BHV-1 antibodies.

Madin Darby bovine kidney (MDBK) cell line

Madin Darby bovine kidney cell line was obtained from the National Centre for Cell Science, Pune. It was maintained in the Virus laboratory by using Dulbecco's modified Eagle's medium (Life technologies, Carlsbad, CA, USA) with 10% foetal calf serum (FCS) (Life technologies) as growth medium. Gentamicin was added in the medium at the rate of 50 mg/l (Life technologies).

Reference virus

The BHV-1 virus isolate (216 IBR II) maintained at the virus laboratory of CADRAD, IVRI was used in this study. In the mSNT, 100 $TCID_{50}$ of BHV-1 was used.

Micro serum neutralization test (mSNT)

All the serum samples were inactivated at 56ºC for 30 min. The serial two fold dilution starting from 1:2 to 1:8 dilutions of serum samples were made in maintenance media (MM) containing 2% FCS. For this, 150 µl of maintenance media was taken in each well of 24-well plates then 150 µl serum sample was added to the one well, mixed properly and 150 µl from this well was transferred to second well and from second well to third well. After proper mixing, 150 µl was discarded from third well leaving final volume to be 150 µl. Now to each well of 24-well plate, 150 µl of BHV-1 containing 100 $TCID_{50}$ was added. Plates were incubated at 37ºC for 2 hours. To each well of 96 well flat bottom plate 100 µl of MDBK cell suspension containing 3×10^4 cells/well was added. 100 µl of each dilution of serum was added to two wells of 96 well plate. All dilutions were tested in duplicate The positive serum control was set up by adding 50 µl of 1:2 dilutions of known positive serum, 50 µl BHV-1 virus and 100 µl cell suspension. In negative serum control well, 50 µl of 1:2 dilution of known negative serum, 50 µl of BHV-1 virus and 100 µl of cell suspensions were added. In virus control well, 50 µl of MM, 50 µl of BHV-1 virus and 100 µl cell suspensions were added. In the cell control well, 100 µl of MM and 100 µl cell suspensions were added. The plate was incubated for 48-72 hours at 37ºC in CO_2 incubator.

Interpretation of result

Cell control wells should show intact monolayer, i.e. no cytopathic effect (CPE). Virus control wells should show CPE. Positive serum control wells should not show CPE. In negative serum control, there should be appearance of

CPE. If the 1:2 dilution serum (final dilution 1:4) does not show CPE, but the 1:4 dilution serum show CPE, the serum was considered positive and the titer is 2. If all wells (1:2 and 1:4 dilutions) show CPE, the serum was considered to be negative.

Competitive ELISA

Commercial competitive ELISA kit procured from Institut Pourquier, France was used for the detection of antibodies to BHV-1. All testing was completed according to the manufacturer's instruction**.**

Percentage inhibition as compared to negative control was calculated using following formula:

$$\text{Inhibition \%} = \frac{\text{OD 450 of analyzed serum}}{\text{Mean OD 450 of negative control}} \times 100$$

Then it is interpreted as: ≤50% (positive), 50- 55% (Suspect) and ≥ 55 % (negative)

Serum with percentage of inhibition ≥ 55% was considered to be from an animal that did not carry specific antibodies to BHV-1. Serum with percentage of inhibition between 50% and 55% was considered as doubtful. Serum with percentage of inhibition ≤ 50% was considered to be coming from an animal that carried specific antibodies to the BHV-1.

Statistical analysis

The sensitivity, specificity, positive predictive value, negative predictive values were calculated and agreement between the tests was evaluated by applying *Kappa* statistic (Thrusfield, 2005).

RESULTS AND DISCUSSION

Out of 200 serum samples tested for antibodies to BHV-1, 30 and 37 samples were found positive by mSNT and cELISA test, respectively. Total 29 samples were positive and 162 were negative to both the tests and 1 sample negative by cELISA was found positive by mSNT. Also, 08 samples negative in mSNT were found positive by cELISA (Table 1). Thus considering SNT as standard test, the relative sensitivity, specificity, positive predictive value, negative predictive value and *Kappa* statistic between SNT and cELISA were determined. Sensitivity and specificity of c-ELISA were 96.67 and 95.29%, respectively (Fig. 1). The positive predictive value and negative predictive value were observed to be 78.38 and 95.29%, respectively. The agreement between the tests was assessed by calculating *Kappa* statitistic and *Kappa* value was observed to be 0.95.

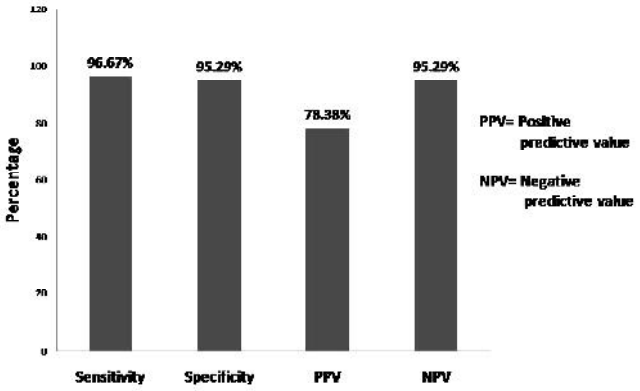

Fig. 1: Evaluation of cELISA relative to mSNT

The VNT has been widely used and is considered to be the 'gold standard' test (Perrin *et al.*, 1994). But there is an urgent need of a test having the properties of ease to perform, rapid, reliable, specific and has the ability to screen large number of samples. In the present study the cELISA was found to have high sensitivity and specificity and the agreement between the two tests was also very good.

Various works have been carried out to evaluate suitability of different ELISA assays in diagnosis of BHV-1 antibodies. Cho and Bohac (1985) developed an ELISA test and found it to be technically superior as a routine diagnostic test in detection of BHV-1 antibodies and reported it to have 100% specificity while testing sera collected from a virus free herd and 100 % sensitivity in detection of antibodies to BHV-1 at six months post vaccination. The ELISA was found to be 100% sensitive in detection of antibodies in sera having neutralizing antibody titers ≥ 1:2. Similarly, Riegel *et al.* (1987) have also described ELISA as a rapid, sensitive, competitive serological test for detecting serum antibodies to bovine herpes virus type-1 with improved sensitivity over the VNT and capable of detecting low level of antibodies earlier post infection.

Table 1: Comparison of competitive ELISA with gold standard serum neutralization test for detection of BHV-1 antibodies

Test		SNT			Sensitivity	Specificity
		Positive	**Negative**	**Total**		
cELISA	**Positive**	29	08	37	96.67%	95.29%
	Negative	01	162	163		
Total		30	170	200		

Kramps *et al.* (1994) developed a simple, convenient blocking ELISA superior to a commercially available indirect ELISA and to the 24-h virus neutralization test in detecting low antibody levels in serum as it had a high sensitivity 99% with a low false-positive rate (0.96) and advocated use of blocking ELISA as test of choice for quicker screening of animals for BHV-1 antibodies so as to minimize any risk of introducing latent BHV1 carriers among noninfected cattle. Kovarick (2001) had developed an ELISA with sensitivity and specificity 99.37% and 97.00% relative to SNT and suggested the use of ELISA in routine diagnosis of infections in place of tedious traditional tests.

Jennifer *et al.* (2009) reported the sensitivity and specificity of blocking ELISA to be 97.7% and 99.4%, respectively as compared with virus neutralization in detection of BHV-1 antibodies with positive predictive value and negative predictive value to be 98.4% and 99.1%, respectively. Neves *et al.* (2009) evaluated three commercial bovine ELISA kits for detection of antibodies against alpha herpesviruses and found them to be highly effective in detection of antibodies to the virus while comparing them with VNT and suggested use of ELISA kit in routine diagnosis as it is cheaper and time saving and thus allowing screening of large number of samples. In an another study, Lyaku *et al.* (1990) observed 98.3% sensitivity and 94.4% specificity, of combined IgG and IgM ELISA relative to SNT. Bashir *et al.* (2011) in a comparison with PCR showed sandwich ELISA to have 91.90 and 93.10%, sensitivity and specificity, respectively. Nandi and Kumar (2011) also evaluated the sensitivity and specificity of cELISA with respect to mSNT and observed higher sensitivity (99.3%) than present study, however specificity and *Kappa* value were observed to be lower than the present work (90.9% and 0.91, respectively).

However, the variation was not appreciable. Das *et al.* (2014) found an avidin-biotin ELISA to have 88.23% sensitivity and high specificity of 95.70% with respect to mSNT In a recent study, Saravanajayam *et al.* (2015) have reported an indirect ELISA to be superior to VNT and observed the sensitivity and specificity of VNT to be 75.16 and 88.24%, respectively as compared to indirect ELISA in detection of BHV-1 antibodies.

CONCLUSION

The results of present study indicate that cELISA is a sensitive and specific test and can serve as an effective, reliable, rapid and simple aid in diagnosis of BHV-1 antibodies as the gold standard test mSNT require, cell culture facility, live virus and need at least 3 days. In India, most of the field laboratories do not have cell culture facilities; hence ELISA particularly cELISA evaluated in the present study can be used extensively for detection of antibodies to BHV-1.

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