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Development of a sandwich ELISA for the detection of bovine herpesvirus type 1

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ABSTRACT

Objective: To develop a standard enzyme-linked immunosorbent assay (ELISA) for the detection of bovine herpesvirus type 1 (BHV-1). **Methods:** The assay was based on hyperimmune rabbit and guinea pig antisera raised against purified BHV-1. Polyethylene glycol precipitation and sucrose density gradient methods were adopted for viral concentration and purification. Antisera were raised using Freund's adjuvant followed by extraction of IgG of high purity. **Results:** Optimum antisera dilutions as determined by titrations were chosen as 1:4 000, whereas the conjugate was used at 1:2 000 dilution. Using 95 clinical specimens, the ELISA test showed a sensitivity and specificity of 91.90 % and 93.10 %, respectively when compared to PCR. The cut-off value was fixed at 0.15 (A_{490}) and a P/N ratio of >1.30 indicated a significant positive reaction. **Conclusions:** The results have demonstrated that this ELISA could efficiently detect BHV-1 and can be used as an important diagnostic tool.

1. Introduction

BHV-1 is the member of the genus varicellovirus in the subfamily *Alphaherpesvirinae*, which belongs to the family *Herpesviridae*. The virus has been associated with a variety of clinical disease manifestations including infectious bovine rhinotracheitis, vulvovaginitis, balanoposthitis, abortions, conjunctivitis and generalized systemic infections[1,2]. The greatest economic impact comes from losses resulting from IBR abortions, which occur chiefly during the last half of gestation, often without evidence of other clinical signs. BHV-1 establishes latency in the sensory neurons of trigeminal ganglia[3]. The main sources of infection are nasal exudates and cough droplets, genital secretions, semen and foetal fluids[4-6]. Diagnosis of BHV-1 infections is mainly carried out by virus isolation, PCR and serology[7-9]. Serum neutralization (SN) test is often used, but it lacks sensitivity, is time consuming and laborious. In this study, we describe the development of a sandwich enzyme-linked immunosorbent (ELISA) which is simple, rapid and reliable test for detecting BHV-1 infections.

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2. Materials and methods

2.1. Virus and cell culture

The reference BHV-1 was isolated from an aborted foetus (7 months) received from a cattle diary farm. Large scale isolation of virus was carried out on Madin Darby Bovine Kidney (MDBK) cell line[10,11]. The confluent monolayers were infected with 1 000 50% tissue culture infective doses ($TCID_{50}$). When the cytopathic effect was observed in 90% monolayer, about 48 hours later, the cultures were frozen at −70 ℃ and thawed at 37 ℃ three times, clarified of cell debris at 1 000 × g for 10 min and supernatant stored at −70 ℃. The pellet was subjected to sonication at 20K amplitude (10 cycles).

2.2. Concentration and purification of virus

The supernatant was clarified by cooling centrifuge at 10 000 × *g* for 30 min. To each 100 mL of the clarified supernatant, 7 g polyethylene glycol 6000 (PEG 6000) and 2.3 g NaCl were added and stored at 4 ℃ overnight[12]. It was then centrifuged at 10 000 × *g* for 30 min, the supernatant was discarded, and the precipitate which contains the virus was suspended in 1/100th volume of TNE Buffer (0.01 M Tris, 0.001 M EDTA and 0.1 M NaCl, pH 7.4) and layered on a 20% − 60% continuous sucrose gradient in

TNE buffer. This was centrifuged at 150 000 × g for 3 hours at 4 ℃ using a Sorvall SW-641 rotor. 1 mL fractions were pooled and assessed for virus concentration by Protein assay (Lowry method)[13] and evaluation of cytopathic effect (CPE) in MDBK cell line in a 96-well plate. The fractions richest in virus were used for hyperimmunization.

2.3. Production of rabbit and guinea pig hyperimmune sera

Two healthy adult New Zealand white rabbits were inoculated intramuscularly with 100 μg of antigen emulsified in complete Freund's adjuvant (CFA; Sigma), followed by three booster doses at weekly intervals with Incomplete Freund's adjuvant (IFA), injection of purified virus without antigen a week later, followed by blood collection one week after last booster[14,15]. Similarly, hyperimmunization was carried out in guinea pigs subcutaneously. Sera were adsorbed overnight at 4 ℃ on an MDBK cell monolayer to remove non-specific antibodies and clarified by centrifug[16]. The titres of rabbit and guinea pig hyperimmune sera were determined by antibody ELISA and were found to be 1: 256 000 and 320 000, respectively. Serum was purified for the extraction of IgG of high purity using a commercially available IgG Purification Kit (GeNei, KT16A).

2.4. ELISA procedure

The dilutions were selected arbitrarily. 96-well microtiter plate (Nunc, Denmark) were coated with 100 μL of 1:1 000 dilution of hyperimmune rabbit (capture) antiserum in coating buffer (0.1 M Carbonate - Bicarbonate Buffer, pH 9.6), and incubated at 4 ℃ overnight. Wells were washed 3 times with PBS-T (0.15% Tween-20) and blocked with 100 μL of 2% BSA (Calbiochem, USA) in PBST for 1 hour at 37 ℃. Wells were washed 3 times with PBS-T and 50 μL of the purified antigen (diluted 1:10 in PBS-T) was added to each well followed by incubation at 37 ℃ for 1 hour. After washing the wells with 3 times PBS-T, 100 μL of 1:1 000 dilution of hyperimmune guineapig (detector) antiserum diluted in PBS-T- BSA was added to each well and the plates incubated at 37 ℃ for 1 hour. After washing the wells 3 times with PBS-T, 100 μL of 1:1 000 dilution of Horseradish peroxidase (HRP) - conjugated rabbit anti-guinea pig immunoglobulin (Dako, Denmark) diluted in PBS-T- BSA was added to each well and the plates incubated at 37 ℃ for 1 hour. Wells were washed 3 times with PBS-T, and then 100 μL of substrate (40 mg o-phenylenediamine) diluted in substrate buffer (40 μL of 30% H_2O_2 in 100 mL of 0.1 M Phosphate-Citrate buffer, pH 5.0) was added to each well and incubated in the dark for 15 min at room temperature. Reaction was stopped by the addition of 50 μL of stop solution (1M H_2SO_4) to each well. The absorbance was measured at 490 nm (A_{490}).

2.5. Determination of optimum dilution of antisera

This was determined by Checkerboard titrations. 8×8 wells ELISA test was performed in order to determine the optimum dilution of rabbit antiserum as capture and guinea pig antiserum as detector. Serial dilutions of hyperimmune rabbit capture antiserum prepared in PBS (1:1000 to 1:128 000) of which 100 μL were added horizontally across the plate to each well so that each row received a single dilution, followed by incubation at 4 ℃ overnight. Wells were washed 3 times with PBS-T, blocked with 100 μL of PBS-T-BSA for 1 hour at 37 ℃, washed again and 50 μL of the purified antigen (diluted 1:10 in PBS-T) added to each well and incubated at 37 ℃ for 1 hour. Wells were washed 3 times with PBS-T, and 100 μL of serial diluted hyperimmune guineapig capture antiserum prepared in PBS (1:1 000 to 1:128 000) were added vertically down the columns to each well so that each column received a single dilution, followed by incubation at 37 ℃ for 1 hour. The plates were then processed as described earlier.

2.6. Determination of optimum dilution of peroxidase conjugate

Serial dilutions of the peroxidase conjugate (HRP-conjugated rabbit anti-guinea pig immunoglobulin) were prepared in PBS-T-BSA (1:500 to 1:6 000). Pre-determined optimum dilutions of capture and detector antisera were used in the ELISA test. The plates were then processed as described earlier.

2.7. Lowest detective concentration of antigen

It was carried out using serial dilutions of the purified virus (1:10 to 1:5120). Optimum dilutions of capture and detector antisera, and conjugate as determined above were used in the ELISA test.

3. Results

Optimum antisera dilutions were determined by checkerboard titrations and the results interpreted graphically (Figure 1) At dilutions of 1:1 000, 1:2 000, 1:4 000 of rabbit capture antisera, the A_{490} values were high (greater than 1.5) with 1:1 000, 1:2 000, 1:4 000 dilutions of guinea pig detector antisera. At higher dilutions (1:8 000, 1:16 000, 1:32 000, 1:64 000 and 1:128 000) of both rabbit and guinea pig antisera, the A_{490} values sharply decreased below 1.5. At dilutions of 1:128 000 for both the antisera, the A_{490} values were less than 0.1. Therefore, dilutions of 1:4 000 were chosen as the optimum working dilutions for both antisera to be used in the preliminary ELISA.

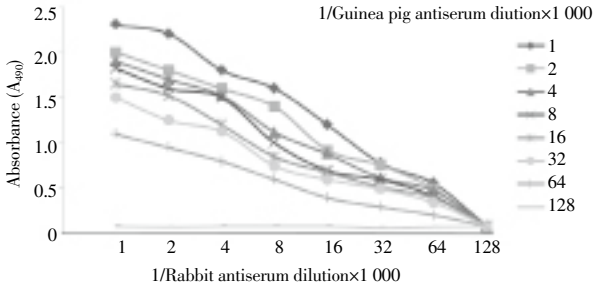

Figure 1. Graph showing optimum dilutions of rabbit and guinea pi antisera measured at antisera A_{490}.
Botthe hyperimmune sera were diluted from 1:1 000 to 1:128 000.

As far as the optimum dilution of peroxidase conjugate, a graph was plotted between the absorbance at 490 nm (A_{490}) and conjugate dilution (Figure 2). At conjugate dilutions of 1:500, 1:1 000, 1:2 000, 1:3 000, there is a plateau curve with A490 values above 1.5 which is in agreement to ELISA requirements. As the dilutions increase (1:4 000, 1:5 000,

and 1:6 000), no plateau curve is obtained, so that the absorbances decrease just from the beginning of the curve. The absorbance values also decrease below 1.0 at higher dilutions. Therefore, conjugate dilutions of 1:2 000 were selected for the preliminary ELISA test.

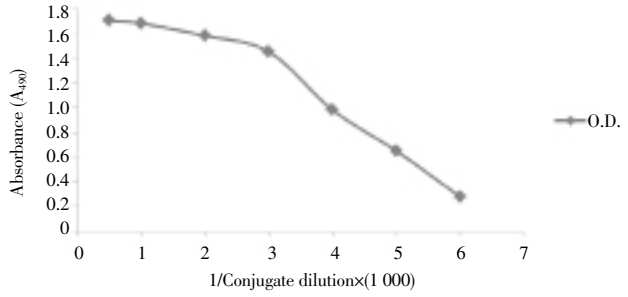

Figure 2. Graph showing optimum dilutions of conjugate. Conjugate was diluted from 1:1 000 to 1:6 000.

For determining the cut-off point, the comparison was made with PCR. Out of total 95 samples tested, 58 PCR negative specimens were used as the negative standard. Cut-off point = arithmetic mean of the OD% of PCR negative samples run in duplicates + three standard deviations, was found to be 0.11 with a standard deviation of 0.011. Therefore, the cut-off value for a positive reaction was fixed as 0.143 ≈ 0.15. The mean positive/negative ratio (P/N ratio) of negative samples was 1.30. A specimen with an A_{490} of >0.15 and a P/N ratio of >1.30 indicated a positive reaction.

The sensitivity and specificity was calculated with respect to PCR (Table 1).

Table 1
Comparison of the results obtained with sandwich ELISA and PCR for BHV-1 detection.

ELISA	PCR		
	Positive	Negative	Total
Positive	34	4	38
Negative	3	54	57
Total	37	58	95

Out of 95 specimens tested by PCR and ELISA, 3 of the 37 PCR positive specimens were found negative in the ELISA. 4 out of 58 specimens, which were negative in PCR, were shown to be positive in ELISA.
Sensitivity (34/34+3) × 100 = 91.90%
Specificity (54/4+54) × 100 = 93.10%

Out of 37 PCR positive specimens, 3 were found to be negative in ELISA. 4 specimens were positive in the ELISA out of 58 PCR negative specimens. Therefore, the sensitivity and specificity of the ELISA was 91.90% and 93.10%, respectively.

The lowest antigen detection concentration, as determined by serial dilutions of the purified antigen, was found at 1:2560 dilution keeping A490 value of 0.15 as cut-off, the value being (0.024 μg/mL).

4. Discussion

The aim of this study is to develop a sandwich ELISA for the detection of BHV-1. In any new ELISA system, the optimal test conditions can be determined by variation of a number of parameters. Rabbit and guinea pig antisera raised against density gradient purified BHV-1 were used as capture and detector antisera, respectively, since the reverse could cause a non-specific binding of conjugate. Antisera were purified for the extraction of IgG of high purity[17,18] as in some cases non-specific reactions due to the IgM fraction necessitate the use of the IgG fractions of the sera[19].

The working dilution of peroxidase conjugate (HRP - conjugated rabbit anti-guinea pig immunoglobulin) was fixed at 1:2000 as higher dilutions could decrease the sensitivity. This selected dilution is in agreement with previous studies where working conjugate dilution was 1:5 000, 1:1 000 and 1:3 000[20-22].

With respect to the cut-off value, sensitivity and specificity, these were determined in comparison to the PCR (adopted as the "golden standard"). Three standard deviations was adopted as the 'rule of thumb' to distinguish negative from positive samples with the cut-off value fixed at 0.15 (A_{490}) and a P/N ratio of 1.30. A criterion for the specimen to be positive in ELISA was established from these data concluding that a specimen with an A_{490} of >0.15 and a P/N ratio of >1.30 indicated a positive reaction. This data favourably compares with previous studies[23-25]. Of the 95 specimens tested, 3 were negative in ELISA out of 37 PCR positive whereas 4 specimens were positive in ELISA out of 58 PCR negative. These results gave an overall sensitivity and specificity of 91.90% and 93.10%, respectively[26,27]. The sensitivity and specificity can be improved by using more purified viral antigen and collection of specimens during the early coarse of the disease in field situations. This ELISA test can be further evaluated with more specimens from field cases during variable intervals of the coarse of disease to determine the detection efficacy of the test. The background values were also low (mean OD of 58 negative specimens = 0.068) when the ELISA test was performed as per the given procedure.

The results indicate that this ELISA is a sensitive, specific and a good tool for the detection of BHV-1. However, it is probable that the detection of low levels of viral antigen can be improved by the evaluation of various critical parameters of ELISA and their optimization. The test is less expensive and time consuming and, therefore, economically suitable and can have extensive application prospectus for IBR diagnosis.

Conflict of interest statement

We declare that we have no conflict of interest.

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References

[1] Gibbs Rweyemamu. Bovine herpesviruses. (Part I, Commonwealth Bureau of Animal Health.) *The Vet Bull* 1977; **47**: 317-343.

[2] Henzel A, Diel DG, Arenhart S, Vogel FSF, Weiblen R, Flores EF. Aspectos virológicos e clínico-patológicos da infecção genital aguda e latente pelo herpesvírus bovino tipo 1.2 em bezerras experimentalmente infectadas. *Pesq Vet Bras* 2008; **28**(3): 140-148.

[3] Jones C, Chowdhury S. A review of the biology of bovine herpesvirus type 1 (BHV-1), its role as a cofactor in the bovine respiratory disease complex and development of improved vaccines. *Anim Health Res Rev* 2007; **8**(2): 187-205.

[4] Schroeder RJ, Moys MD. An acute upper respiratory infection of dairy cattle. *J Am Vet Med Assoc* 1954; **125**: 471-472.

[5] Van Oirschot JT, Kaashoek MJ, Rijsewijk FA, Stegeman JA. The use of marker vaccine in eradication of herpesviruses. *J Biotechnol* 1996; **44**: 75-81.

[6] Lata J, Kanani AN, Purohit JH, Joshi CG, Rank DN, Vinay K. Detection of bovine herpesvirus 1 (BHV-1) infection in semen of Indian breeding bulls by polymerase chain reaction (PCR) and its characterization by DNA sequencing. *Buffalo Bull* 2009; **28**(2): 76-84.

[7] Grom J, Hostnik P, Toplak I, Barlic-Maganja D. Molecular detection of BHV-1 in artificially inoculated semen and in the semen of a latently infected bull treated with dexamethasone. *Vet J* 2006; **171**: 539-44.

[8] Gupta P K, Saini M, Rai A. Rapid and sensitive PCR-based test for detection of bovine herpesvirus-1 in semen. *Indian J Virol* 2006; **17**: 23-27.

[9] Madbouly HM, Tamam SM, Abd-El-Gaid AM. Isolation and identification of bovine herpesvirus-1 (BHV-1) from semen of foreign breeds bulls. *BS Vet Med J* 2008; **18** (2): 22-27.

[10] Intisar KS, Ali YH, Khalafalla AI, Rahman Mahasin EA, Amin AS. Natural exposure of Dromedary camels in Sudan ti infectious bovine rhinotracheitis virus (bovine herpesvirus-1). *Acta Tropica* 2009; **111**(3): 243-246.

[11] Sandra P, Florencia M, Kazima S, Alan D, Clinton J. Premature expression of the latency-related RNA encoded by bovine herpesvirus type-1 correlates with higher levels of beta interferon RNA expression in productively infected cells. *J Gen Virol* 2008; **89**: 1338-1345.

[12] Abd El - Hafeiz YGM, Abou Gazia KAA, Ibrahim IGA. Sero prevalence of bovine viral diarrhea virus and bovine herpesvirus -1 infection in Egypt and their relation to Brucellosis. *Global Veterinaria* 2010; **4**: 1-5.

[13] Lowry OH, Rosenbroug NJ, Farr AL, Randall RJ. Protein measurement with Folin phenol reagent. *J Biol Chem* 1951; **193** (1): 265-275.

[14] Ausubel FA, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, et al (eds). *Current protocols in molecular biology*. New York: John Wiley & Sons; 2000.

[15] El Hussein AM, Kamel I, Ali YH, Fadol MA. Prevalence of antibodies to infectious bovine rhinotracheitis virus in Sudanese cattle. *J Sci Technol* 2005; **6** (1): 151-157.

[16] Deka D, Ramneek, NK Maiti, Oberoi MS. Detection of bovine herpesvirus-1 infection in breeding bull semen by vius isolation and polymerase chain reaction. *Rev Sci Tech Off Int Epiz* 2005; **24** (3): 1085-1094.

[17] Winkler MTC, Osorio FA, Sondahl MS, Filho FB, Barahona H. Monoclonal antibodies against bovine herpesvirus type-1. *Bol Centr Panan Fiebre Aftosa* 1992; **58**: 35-43.

[18] Zhao Y, Zuo Y, Zhang L, Fan J, Yang H, Qin J. Development of a sandwich Dot-ELISA for detecting bovine viral diarrhea virus antigen. *Front Agric China* 2009; **3**(3): 325-331.

[19] Yolken RH. Enzyme- linked immunosorbent assay (ELISA): A practical tool for rapid diagnosis of viruses and other infectious agents. *The Yale J Biol Med* 1980; **53**: 85-92.

[20] Jiang XI, Cubitt D, Hu J, Dai X, J Treanor, Matson D, et al. Development of an ELISA to detect MX virus, a human calcivirus. *J Gen Viro* 1995; **76**: 2739-2747.

[21] Singh RP, Sreenivasa BP, Dhar P, Shah LC, Bandyopadhyay SK. Development of a monoclonal antibody based competitive - ELISA for detection and titration of antibodies to peste des petits ruminants (PPR) virus. *Vet Microbiol* 2004; **98**: 3-15.

[22] Botus D, Oncescu T. Optimizing immunoenzymatic reactions (ELISA) for the detection of antibody against Newcastle Disease virus. Analele Universitatii din Bucuresti- Chimie Anul XV (serie noua). 2006; vol II: 33-41.

[23] Collins JK, Butcher ANN C, Teramoto YA, Winston S. Rapid detection of bovine herpesvirus type -1 Antigens in nasal swab specimen with an antigen capture enzyme - linked immunosorbent assay. *J Clin Microbiol* 1985; **21** (3): 375-380.

[24] Hansman GS, Guntapong R, Pongsuwanna Y, Natori K, Katayama K, Takeda N. Development of an antigen ELISA to detect Sapovirus in clinical stool specimens. *Arch Virol* 2006; **151**: 551-561.

[25] Quinting B, Robert B, Letellier C, Boxus M, Kerkhofs P, Schynts F, et al. Development of a 1-step enzyme-linked immunosorbent assay for the rapid diagnosis of bovine respiratory syncytial virus in postmortem specimens. *J Vet Diagn invest* 2007; **19**: 238-243.

[26] Kovarcik K. The development and application of an indirect ELISA test for the detection of antibodies to bovine respiratory syncytial virus in blood serum. *Vet Med-Czech* 2001; **46** (2): 29-34.

[27] Godhardt-Cooper JA, Zoromski J, Toohey-Kurth K. Evaluation of a blocking enzyme-linked immunosorbent assay for serological diagnosis of Bovine herpesvirus 1. *J Vet Diagn Invest* 2009; **21**: 523-526.