Blood or Serum Collected on Filter Paper for Detection of Antibodies to Bovine Herpesvirus Type 1 (BoHV-1)*

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ABSTRACT

Background: The method of collection as well as the packaging conditions in which samples are submitted to laboratories play a critical role on the acquisition of reliable results on diagnostic tests. Alternative methods however have been proposed, as the adsorption of blood or serum in filter paper. In this work, it was evaluated the viability of using serum or whole blood samples from bovines collected in filter paper for serological testing against bovine herpesvirus type 1 (BoHV-1).

Materials, Methods & Results: One hundred and seven whole blood and serum samples were collected by standard methods. Serum neutralization test was used as a golden standard method for evaluation of the serum samples. The same samples of both whole blood and sera were also adsorbed on filter paper strips for further comparisons. Optimal conditions for serum and blood elution from filter paper were determined. Adsorbed samples on filter paper disks were eluted in PBS and subsequently diluted further with PBS 5% skimmed milk. The eluates were tested for antibodies to BoHV-1 in an indirect ELISA (iELISA) and matched with the results obtained by serum neutralization of standard serum samples. Comparison between results obtained by serum neutralization of standard serum samples and the ones from the iELISA of serum eluted from paper disks resulted in sensitivity, specificity, positive and negative predictive values of 95, 94, 80, 99%, respectively, and a correlation coefficient (κ) of 0.83. Comparison between the results of serum neutralization of standard serum samples and the ones from the iELISA of blood eluted from paper disks resulted in sensitivity, specificity, positive and negative predictive values of 91, 97, 87, 98%, respectively, and a correlation coefficient (κ) of 0.86. Standard serum samples were also tested in the iELISA and the results compared with those of iELISA from samples eluted from filter paper. Comparison of the results from iELISA between serum samples and serum eluted from filter paper resulted in sensitivity, specificity, positive and negative predictive values of 91, 81, 69, 95%, respectively, and a correlation coefficient (κ) of 0.66. The comparison of iELISA results between standard serum and blood samples eluted from filter paper resulted, in sensitivity, specificity, positive and negative predictive values of 78, 92, 82, 89%, respectively, and a correlation coefficient (κ) of 0.70. Fifty samples collected on filter paper were retested eight months later in order to determine whether those would retain its viability; both sensitivity and specificity remained unaltered.

Discussion: Sampling on filter paper has been successfully described for antibody detection in a number of diseases such as Aujeszky's disease virus and Newcastle disease virus. In this work, it has been demonstrated that both blood and sera collected in filter paper can be used for submission of samples aiming detection of antibodies to BoHV-1 in an iELISA, without significant loss of sensitivity and specificity. Submission of samples on filter paper is a practical and economical alternative as no special conditions are required for storaging and transporting. This method also enables the collection of samples from distant places assuring its quality for serological tests.

Keywords: serum neutralization, indirect ELISA, bovine herpesvirus, filter paper adsorbed samples.

INTRODUCTION

Serological tests are essential tools in any diagnostic laboratory [1,17]. However, the quality of the sample is essential for reliable results. If samples have to be sent through long distances, particularly in hot climates, not only the quality of the collected sample but also its packaging conditions may play a critical role on the final results obtained. In diagnostic laboratories, inadequately packaged, contaminated or "lost" samples (due to transport difficulties) are day to day reality. In view of these problems, several researchers have proposed alternatives to substitute the standard serum samples submitted in glass vials with rubber stoppers within an ice–containing box. Some of these alternatives included blood adsorbed on filter paper, from which antibodies could then be eluted and subsequently detected in immunoassays [2,5,7,12]. Adaptations of this procedure have been applied to the serological diagnosis of various infections for more than twenty years [2,4,15]. In Brazil, a large country where the hot climate is dominant, the problems of sample conservation and submission to the laboratory are always present. Therefore, tests that could rely on sample submission without the need for ice or any special container would be most advantageous. In view of that, aiming to facilitate collection, storage and posting of samples to the laboratory, this study was conducted to evaluate the performance of serum and blood collected on filter paper for the detection of antibodies to BoHV-1 in an indirect enzyme-linked immunosorbent assay (iELISA).

MATERIALS AND METHODS

Cells and viruses

Madin-Darby bovine kidney cells (MDBK) were cultivated in Eagle's Minimum Essential Media (E-MEM)[1] supplemented with 10 % fetal bovine sera[2]. Cells were multiplied and maintained following standard procedures [20].

The bovine herpesvirus type 1 (BoHV-1) strain EVI 123/98 was isolated at the Laboratory of Virology, Instituto de Pesquisas Veterinárias Desidério Finamor (IPVDF) [11].

Serum and blood samples

Whole blood samples were collected from 107 adult animals from the laboratory herd. Whole blood samples were collected from the jugular vein and allowed to diffuse to saturation onto 2.5 cm X 10 cm filter paper strips (Whatman grade no.3). For serum collection, blood samples were collected on glass tubes without anti-coagulant, allowed to clot for 1 h at 37°C and kept for 16 h at 4°C. The serum was separated and stored at -20°C until use. The sera were then allowed to adsorb to saturation onto filter paper strips. After adsorption, filter paper strips were air-dried at room temperature for 4 h and sealed in plastic bags. Additionally, serum samples were collected from five calves immunized with a BoHV-1 vaccine and used as positive controls. Serum samples from five animals not showing antibody response to BoHV-1 on serum neutralization tests were used as negative control.

Elution of antibodies from samples on filter paper

Filter paper discs (0.6 mm diameter) were cut from the filter paper strips impregnated with blood or serum, with the aid of an ordinary paper punch. The volume adsorbed on the filter paper disc was determined by saturation with known amounts of serum or blood. Each disc was shown to adsorb 5 μL of either serum or blood. For elution, best conditions were achieved with one (for blood) or two (for serum) discs placed in microtubes containing 100 mL of phosphate buffered saline (150 mM NaCl; 8.7 mM $Na_2HPO_4.2H_2O$; 1.6 mM $NaH_2PO_4.H_2O$) plus 0.05% Tween 20 (PBS-T20) and kept under gentle shaking for 1 h at room temperature. For further dilution of samples, 100 μL of PBS-T20 with 5% skimmed milk were added to each eluate. Sera or blood samples prepared this way were assumed to be diluted 1:40 (blood) or 1:20 (serum). The dilution of choice was the one that showed a significant difference between the mean optical density (OD) of positive control sera divided by the mean OD of the negative control sera (not shown).

Serum neutralization tests

Serum neutralization tests (SN) were performed as previously described [6] with modifications. Fifty microlitre volumes of standard serum samples were added, in quadruplicate, to 96-well cell culture microplates[3], and subsequently diluted in twofold steps (from 1:2 to 1:128). Next, one hundred tissue culture 50% infective doses ($TCID_{50}$) of BoHV-1 strain EVI 123/98 were added to serum dilutions in 50 μL volumes. The serum/virus mixture was incubated for 1 h at 37°C. Subsequently, 50 mL of an MDBK cell suspension containing 2 x 10^4 cells were added to each well and incubated for 1 h, at 37°C in a 5% CO_2 atmosphere. Appropriate controls (positive serum, negative serum, cell

control wells) were added to each test. Search for characteristic cytopathic effect (CPE) was performed after 24, 48 and 72 h of incubation and final readings were performed after 96 h of incubation. Samples displaying titres $= 10^2$ TCID$_{50}$ were considered positive.

Indirect ELISA (iELISA)

A stock iELISA antigen was prepared on MDBK cells infected with BoHV-1 strain EVI 123/98 and further treated with 0.2% n-octyl-glucopyranoside (OGP) following previously described methods [21]. Antigen-coated plates prepared as above were washed three times with 100 mL PBS-T20. One hundred microlitres of each sample eluate (sera or blood) or standard serum samples were added to duplicate wells and plates incubated for 1 h at 37°C. Subsequently, plates were washed three times with PBS-T20 and an anti-bovine IgG/peroxidase conjugate[4] diluted as appropriate (1:10.000 in PBS-T20) added to wells. The incubation/washing steps were repeated as above and followed by addition of 5 mL of ortho-phenylenediamine[4] (OPD) in citrate-phosphate buffer (1.8 mM OPD, 53 mM citric acid, 130 mM Na$_2$HPO$_4$, pH 5.0) and 30 mL of 30% H$_2$O$_2$. Color development was stopped by the addition of 2 M H$_2$SO$_4$. The optical density (OD) was measured spectro-photometrically at 450 nm in a Titertek Multiskan ELISA reader.

Fifty samples of serum and blood adsorbed onto filter paper were kept at 4°C and eight months later tested again in the iELISA in order to evaluate the effect of long-term storage on the sensitivity and specificity of the test.

Statistical methods

Results obtained with eluates of serum or blood collected on filter paper were compared to those obtained with the corresponding sera collected by standard procedures.

The cutoff point of the iELISA was calculated by the following formula:

Cut off $= \mu DO + 1.76\sigma$, where μDO correspond to the arithmetic mean OD% obtained with seronegative control sera, plus 1.76 standard deviation, "σ". The standard deviation plus or minus 1.76 was chosen to include 92.2% of the true negative samples [3] .

The assays were validated by calculating the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) with the DAG STAT program (http://www.mhri.edu.au/biostats/DAG_Stat/). The correlation coefficient between the techniques was calculated by the Kappa method (http://www.mhri.edu.au/biostats/DAG_Stat/).

RESULTS

Serum neutralization (SN) results revealed 85 negative and 22 positive serum samples. Samples obtained from filter paper eluates were evaluated in iELISA and the results compared to those of the respective standard sera obtained by SN. The comparison between SN and filter paper serum eluates are shown in Table 1. Those between SN and filter paper blood eluates are shown on Table 2. Both serum and blood filter paper eluates showed high sensitivities of 95% and 91%, respectively, and specificities of 94% and 97%, respectively when

Table 1. Comparison between the results of iELISAs performed on serum eluted from filter paper (iELISA serum-FP) and on standard serum samples in serum neutralization tests (SN).

iELISA Serum-FP		
SN	Positive	Negative
Positive	20	2
Negative	5	80

Validity analysis: sensitivity = 95%; specificity = 94%; PPV: 80%; NPV: 99%; k: 0,83.

Table 2. Comparison between the results of iELISAs performed on blood eluted from filter papel (iELISA blood-FP) and on standard serum samples in serum neutralization tests (SN).

iELISA Blood-FP		
SN	Positive	Negative
Positive	20	2
Negative	3	82

Validity analysis: sensitivy: 91%; specificity: 97%; PPV: 87%; NPV: 98%; k: 0,86.

comparing iELISA and SN results. Moreover, the correlation indexes (κ) between SN and iELISA results of these samples were considered almost perfect as shown in Tables 1 and 2.

Standard serum samples were also tested in the iELISA and the results compared to the ones from serum and blood eluates tested in the iELISA (Tables 3 and 4, respectively). Both serum and blood eluates gave rise to sensitivities of 91 % and 78 %, respectively, and specificities of 81 % and 92 %, respectively, when comparing iELISA results. The long term repeatability of the filter paper ELISA test was evaluated on 50 samples of serum and 50 samples of blood collected on filter paper. This experiment revealed that the sensitivity and specificity of the iELISA remained unaltered for both serum and blood eluates after eight months storage of filter paper strips at 4°C, indicating that long term storage had no harmful effect on the results of the iELISA.

Table 3. Comparison between the results of iELISAs performed on serum eluted from filter paper (iELISA Serum-FP) and on standard serum samples (iELISA Serum).

iELISA Serum	iELISA Serum-FP	
	Positive	Negative
Positive	31	3
Negative	14	59

Validity analysis: sensitivity: 91%; specificity: 81%; PPV: 69%; NPV: 95%; k: 0,66.

Table 4. Comparison between results of iELISAs performed on blood absorbed onto filter paper (iELISA Blood-FP) and on standard serum samples (iELISA Serum).

iELISA Serum	iELISA Blood-FP	
	Positive	Negative
Positive	28	8
Negative	6	65

Validity analysis: sensitivity: 78%; specificity: 92%; PPV: 82%; npv: 89%; k: 0,70

DISCUSSION

In this study, 107 sera and blood samples collected on filter paper strips were tested in iELISA and compared to standard serum samples analyzed by SN and iELISA. Our main goal was to check the paper strip sampling method and the most appropriate specimen (serum or blood) to be adsorbed and used as source for antibodies for further laboratory testing. Sera were first analyzed by SN which was taken as a gold standard method. The results obtained revealed that eluted samples from serum or blood collected on filter paper strips perform quite well in comparison with serum samples collected by usual methods. The steps necessary to perform the iELISA did not vary, with the exception of the additional handling needed for elution of antibodies from the impregnated filter paper. Caution was taken to ensure that the elution steps would be adjusted to decrease the background so as to avoid elevated background noise as reported elsewhere [7]. Among the measures taken to minimize such noise, eluates were diluted in PBS-T20 and 5% fat free milk powder, what provided a good signal-to-noise ratio. With the process adopted here, haemolysis did not seem to interfere with testing as demonstrated by the results obtained with blood eluted from filter paper.

The advantages of sample submission on filter paper have already been highlighted in a number of occasions [2,7,10]. The method has been successfully applied at the detection of antibodies to Chagas' disease [16], hepatitis B [13], hepatitis A [9], human immunodeficiency virus (HIV) [5,19], measles [7,10,12], Aujeszky's disease virus [4], Newcastle disease virus [14] and many others. Here, it has been demonstrated that blood or sera collected in filter paper can be used for submission of samples aiming detection of antibodies to BoHV-1 in an iELISA without significant loss of sensitivity and specificity. Filter paper adsorbed paper eliminates the need for dealing with large volumes of blood or serum, making contamination unlikely, as also pointed out by others [8]. In addition, filter paper is much more convenient for storaging, transporting or sending/posting. Satisfactory storage of samples adsorbed onto filter paper have been demonstrated in some reports for up to 1 year at 4°C [10,18] or several days at room temperature [10,13]. In this study, the reproducibility of

the filter paper ELISA test was evaluated after 8 months at 4°C, with no alteration in the test´s sensitivity or specificity.

CONCLUSION

In the present study, whole blood or sera collected on filter paper were shown to be adequate sources of antibodies for laboratory testing in assays such as the iELISA employed here, aiming detection of antibodies to BoHV-1. Sample collection is simpler, more practical and economical than the usual blood/serum collection in vials; in addition, filter paper-collected specimens do not require refrigeration for transporting to the laboratory. This method of sample collection seems attractive for submitting specimens, particularly when the laboratory is distant from the farm. Blood or serum samples collected this way may in principle be used for the detection of antibodies to various other infectious diseases, although test conditions should be standardized in each particular case.

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