

Dot-Blot Enzyme Immunoassay for the Detection of Bovine Herpes Virus-1(BHV-1) Antibodies

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Abstract: Various detection methods have been developed for serological monitoring BHV1, an alphaherpesvirus and the cause of the respiratory disease, abortion, conjunctivitis and other clinical forms of disease complex in cattle, in different laboratories throughout the world. The objective of this study was to develop and evaluate dot-blot enzyme immunoassay as a proper and inexpensive method for detection of BHV-1 antibodies in cattle. In this assay, a whole particle of a field isolated of BHV-1, identified by PCR and VNT and was used as an antigen. A total of 360 bovine sera in three different age groups with known ELISA titer for BHV-1 antibodies and rabbit anti cow Ig HRP conjugated (Dako, Denmark) were used as primary and secondary antibody, respectively. 4-chloronaphthol (Merk, Germany) was used as substrate. The concentrations of HRP-conjugated, sera and antigen were optimized. Statistical analysis showed sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Dot-blot enzyme immunoassay were 86.4%, 92.7%, 94.4% and 84.4% respectively.

Key words: Dot-blot enzyme immunoassays • Bovine herpesvirus-1 • ELISA • PCR • Virus neutralization test

INTRODUCTION

The bovine herpesvirus type-1 or the infectious bovine rhinotracheitis (IBR) virus is an alphaherpesvirus and the cause of the respiratory disease, abortion, conjunctivitis and other clinical forms of disease complex. Genetic analyses of various clinical isolates have found at least three distinct BHV-1 subtype: a respiratory subtype, a genital subtype and an encephalitic subtype designated as BHV-1.1, BHV-1.2 and BHV-1.3, respectively. [1-3].

IBR occurs on all continents, although there are differences in prevalence and incidence. Immunity against BoHV-1, either natural or induced following vaccination, has been reported to protect from clinical disease as well as from the negative consequences attributed to systemic spread of the virus [4-9]. Many countries have implemented monitoring and eradication programs. In order to serological monitoring and during and after an eradication campaign, annual surveillance by serological means is the most important prerequisite for

early recognition of newly imported cases and for maintaining the IBR-free status. Since wild ranging animals as well as sheep and goats can be excluded as BoHV-1 reservoirs, movement of seropositive cattle and trade with BoHV-1-positive semen used in artificial insemination are to be considered as the most important ways to reintroduce the virus into IBR-free facilities [10, 11]. Various detection methods have been developed for monitoring BHV1 in different laboratories throughout the world. The virus neutralization test enzyme-linked immunosorbent assays (ELISA) are most widely used for antibody detection. Each of these methods may have disadvantages in terms of sensitivity, specificity, cost and convenience. None of them, however, is simple enough for field application by relatively untrained personnel [12].

The objective of this study was to develop and evaluate a proper and inexpensive system for detection of seropositive cows and estimation of the sensitivity and specificity of the newly-designed test for detection of BHV-1 antibodies in cattle.

MATERIALS AND METHODS

Sampling: A total of 360 sera were compiled from twenty-eight herds, in three different age groups. And they were tested for BHV-1 antibodies by ELISA test.

ELISA Test: The antibodies to BHV-1 were detected using a commercially available ELISA kits developed by Svanova Biothech (Uppsala, Sweden).

Virus Culture: The isolate of BHV-1 was inoculated by MOI (0.01 - 0.1) into the IRKHBK cell line in Dulbecco's modified Eagle's medium containing 3% Fetal Bovine Serum (FBS).

Antigen Preparation: In this assay, a whole particle of a field isolate of BHV-1 identified by PCR and Virus Neutralization Test (VNT), it was used as antigen. Virus was inoculated into the (IRKHBK, NCBI Code: C541) cell line. Following three cycles of rapid freezing and thawing, the mixture of cell debris and medium was clarified by centrifugation. The pellet was then resuspended in a small volume of phosphate buffered saline (PBS) and stored in aliquots at -70 °C.

PCR: Briefly, DNA of virus was extracted by Invitex Extraction of DNA. Next a part of gI of BHV-1 was amplified by Sina Gen PCR Master Kit. The production of PCR was appeared by irritating of X ray on Agarose gel.

VNT: This test was done according to OIE procedure, briefly, Inactivate sera, including control standard sera, for 30 minutes in a water bath at 56°C. Make doubling dilutions of test sera in cell culture medium. Start with undiluted serum and continue to 1/1024 horizontally in a 96-well flat-bottomed cell-culture grade microtitre plate, at least three wells per dilution and 50 µl volumes per well. Dilutions of a positive control serum and of weak positive and negative internal control sera, are also included in the test. An extra well with undiluted test serum is used for toxicity control of sera. Add 50 µl per well of BoHV-1 stock at a dilution in culture medium calculated to provide 100-200 TCID₅₀ per well. In the toxicity control wells, add 50 µl of culture medium in place of virus. Add 100 µl of culture medium to ten empty wells for cell controls. Make at least four tenfold dilutions of the residual virus stock (back titration) in culture medium, using 50 µl per

well and at least four wells per dilution. Incubate the plates for 24 hours at 37°C. Add 100 µl per well of the cell suspension at 3×10^4 cells per well. Incubate the plates for 3-5 days at 37°C. Read the plates microscopically for CPEs [12, 13].

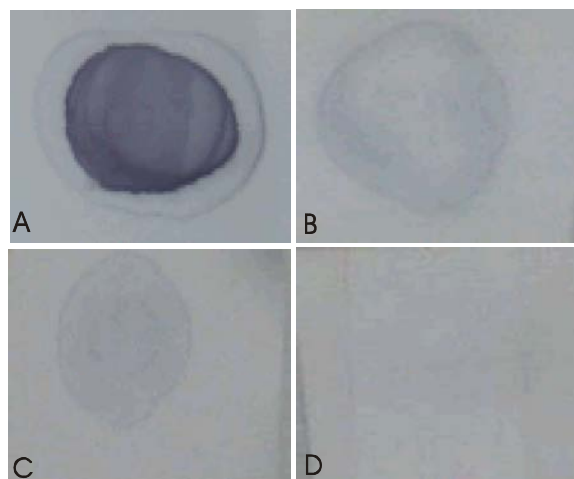
DB-EIA: Instrumental concentrations of HRP-conjugated, sera and antigen were optimized by a checker board. All 360 sera were tested by following procedure. Firstly, Viral antigen prepared as described above was spotted on to 1.5×1.5 cm nitrocellulose (NC) membranes at 20 µL per spot and then allowed to air dry. Next, Each NC membrane was transferred into a well of a 6 well flat bottom, cell culture plate (utilized and washed) and incubated in blocking solution (1% non fat dry milk in TBS) for 1 h at room temperature with occasional hand shaking. The membranes were then washed for 3×5 min in TTBS, overlaid with optimized dilution of sera and incubated for 1 h at 37°C and washed 3×5 min in TTBS. All the membranes were overlaid with HRP conjugated (Dako, Denmark) incubated and washed as described before and developed using 4-chloronaphthol (Merk, Germany) as substrate. The color reaction was allowed to proceed for 10 min and then stopped with several washes with distilled water. The membranes were air dried in the dark before being read. Two tests (ELISA and DB-EIA) were read independently and blind to the results of another test [14, 15].

Statistical Analysis: Two tests (ELISA and DB-EIA) were read independently and blind to the results of another test [14, 15]. Sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Dot-blot enzyme immunoassay were detected [16].

RESULT

Results of VNT and PCR confirmed virus was BHV-1. Following optimization of DB-EIA the optimal concentrations for HRP-conjugated and sera were 1:500 and 1:40 respectively. The best result of checker board was with 20 µl of 108 virus.

Positive samples were visually determined by the appearance of a dark spot (Fig. 1A) at the site where the antigen was spotted while minor/no color change (Fig. 1. B, C and D) was considered a negative result [17, 18].



- A Undiluted positive serum with BHV-1 antigen,
 B Undiluted positive serum with negative control antigen,
 C Undiluted negative serum with BHV-1 antigen
 D Undiluted negative serum with negative control antigen

Fig. 1: Examples of DB-EIA results:

Table 1: Results of ELISA and DB-ELA tests.

	ELISA+	ELISA-	
DB-ELA+	185	11	196
DB-ELA-	26	138	164
	211	149	360

Statistical analysis sensitivity, specificity, Positive Predictive Value (PPV) and Negative Predictive Value (NPV) of Dot-blot enzyme immunoassay were 87.6%, 92.7%, 94.4% and 84.2% respectively.

DISCUSSION

The most important factors influencing sensitivity are antigen concentration and sera dilution. As a conclusion, DB-EIA is equivalent to ELISA and SN in terms of sensitivity and specificity. The specificity level of the DB-EIA assay found in this study allows its usage as an alternative method for SN or plate ELISA. The high sensitivity of the DB-EIA method makes it a suitable screening test to evaluate herd immunity and to perform serodiagnosis (19, 20). In addition to its superior performance with the panel of tested sera used in this study, the DB-EIA was much easier to read and interpret than ELISA and SN tests and unlike the SN method, DB-EIA described here, does not require

pretreatment of samples or expensive supplies and equipment (e.g., cell culture equipments). A possible disadvantage of DB-EIA, compared to SN or ELISA tests, is that it does not detect antibody titer or optical density as a document [15].

The present study provides evidence that it is still possible to implement simple techniques for the sero-monitoring of BHV-1 which offer high sensitivity and specificity. Where costs are an issue, and in the absence of more elaborated diagnostic tests in many developing countries, simple tests such as DB-EIA could still deliver reliable information that can be used to monitor individuals and/or herds that have problems with BHV-1 infections.

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