VETERINARSKI ARHIV 79 (4), 343-350, 2009

Dot-blot enzyme immunoassay for the detection of bovine viral diarrhea virus antibodies

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HEMMATZADEH, F., F. AMINI: Dot-blot enzyme immunoassay for the detection of bovine viral diarrhea virus antibodies. Vet. arhiv 79, 343-350, 2009.

ABSTRACT

Dot-blot enzyme immunoassay (DB-EIA) was utilized for the detection of bovine viral diarrhea (BVD) antibodies in infected cattle. In this assay whole particles of the NADL strain of BVD virus were used as an antigen. A total of 1250 sera from SN and ELISA tested cows were tested for BVDV antibodies. HRPO-conjugated protein G was used to detect bound BVDV antibodies using TMB as the substrate. Statistical analyses did not show any significant differences in the sensitivity of DB-EIA, to the ELISA and SN tests. The assay proved to be a simple, inexpensive, reliable and rapid tool for BVD serodiagnosis.

Key words: dot-blot enzyme immunoassays, bovine viral diarrhea, serum neutralization test, ELISA

Introduction

Bovine viral diarrhea virus (BVDV), a member of the genus *Pestivirus*, causes a range of symptoms in cattle, leading to widespread economic losses worldwide. Postnatal infection is transient and followed by development of long lasting antibodies (PATON, 1995). Prenatal infection can also result in immunotolerance and persistent infection (PI) in calves (NETTLETON and ENTRICAN, 1995; STOKSTAD et al., 2003).

Animals persistently infected (PI) with BVDV are regarded as the major source of spread of infection within and among herds (LONERAGAN et al., 2005). In addition, the presence of PI animals is known to cause sero-conversion in other animals within herds (PATON, 1995; BOLIN and GROOMS, 2004).

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ISSN 0372-5480
Printed in Croatia

Various detection methods have been developed for monitoring BVD in different laboratories throughout the world. The most extensively used protocols for serodiagnosis of BVD are based on the plate ELISA and serum neutralization (SN) methods (BROCK et al., 1986; HOUE et al., 1995). 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 (VILCEK et al., 1994; FREDRIKSEN et al., 1999; SAINO et al., 1994; BARLIC-MAGANJA and GROM, 2001).

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

Materials and methods

Sampling. A total of 1250 sera were collected from various regions in Iran from twenty-eight herds, all with a history of BVDV infection confirmed by antigen capture ELISA and SN tests. These also included 809 individual SN negative sera which were used as controls.

SN and ELISA tests. For virus neutralization test, the sera were initially heat-inactivated, antibody titers were measured against the NADL strain of the BVD virus using the micro-titration method. In brief, serial twofold dilutions (starting from ½ ending to 1/128) of sera in duplicate were prepared in 50 µL of Dulbecco's modified Eagle's medium (Sigma-Aldrich Germany) and mixed with 50 µL of the virus suspension containing 100 $TCID_{50}$ of BVD (NADL). After incubation for 1 h at 37 °C in a humidified atmosphere containing 5% CO_2, 200,000 MD-BK cells in a total volume of 50 µL were added to each well of 96 tissue culture plates. The plates were then incubated at 37 °C for 3 days, and monitored periodically for the development of CPE. The highest serum dilution that neutralized virus infectivity was considered as the serum titer. Serum samples with a titer of 1/4 or higher were considered as positive (MURPHY et al., 1999; HAY et al., 2002).

All serum samples were also analyzed using an indirect antibody enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer's (Svanova Biotech, Uppsala, Sweden) instructions (NISKANEN et al., 1991).

Antigen preparation. The NADL strain of the BVD virus was inoculated into the MD-BK cell line (previously tested for BVD virus by PCR and FA tests) in Dulbecco's modified Eagle's medium containing 3% Fetal Bovine Serum (FBS) (Gibco, BVD antibody and antigen free; based on the manufacture's claim and ELISA test for antibody) and harvested when the cytopathic effects (CPE) were well advanced. Following three cycles of rapid freezing and thawing, the mixture of cell debris and medium was clarified by centrifugation at 600g for 20 min, and ultra-centrifuged at 70,000 g for 3 h through a 30% sucrose cushion. The pellet was then resuspended in a small volume of phosphate

buffered saline (PBS), and stored in aliquots at -80 °C. The protein content of the prepared antigen was measured by Bradford method (HAY et al., 2002) and adjusted to 2 mg/ml. Uninfected cell cultures were processed similarly to be used as negative control for antigen.

DB-EIA. Viral antigen prepared as described above was spotted onto 1×1cm nitrocellulose (NC) membranes (Schleicher & Schuell BA-S 83, Keene, NH) at 20 µL per spot and then allowed to air dry. Antigen spotted membranes were stored in a fridge and utilized for the test over a 6 month period. To optimize the DB-EIA and to determine the best concentration of antigen and antibodies checkerboard titration, the previously tested positive and negative antisera were each tested against different dilutions of coating antigens.

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 (3% BSA/0.05% Tween-20/PBS) for 1 h at room temperature with occasional hand shaking (ROY and VENUGOPALAN, 1999).

The membranes were then washed for 3×5 min in PBS-T, overlaid with two-fold serial dilutions (1/2 to 1/128) of 100 positive and 100 negative sera previously tested by ELISA and SN tests separately and incubated for 1 h at room temperature and washed 3×5 min in PBS-T. All the membranes were overlaid with horseradish peroxidase (HRP) conjugated protein G (Sigma, chemical. Co. St Louis, Mo.), incubated and washed as described before and developed using TMB (3,3', 5,5'-tetramethylbenzidine) as substrate solution (Sigma, Chemical. Co. St Louis, Mo.). 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. Positive samples were visually determined by the appearance of a blue 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. (CHANG et al., 1998; LU et al., 1996).

Each dot was scored independently as being reactive or non-reactive and was subsequently compared with values of ELISA and SN tests for analysis (CESAR et al., 2000). After optimization of the method, all 1250 sera samples were examined by the DB-EIA method as previously described. All three tests were read independently and blind to the results of the other tests by different technicians.

Statistical analysis. The kappa statistic and apparent correlation rate were used to measure the strength of agreement between the results of the tests by the ELISA, SN and DB-EIA tests. A kappa statistic value of >0.75 represents excellent agreement between the results (ENOE et al., 2000, MARTIN et al., 1987)

Results

Following optimization of DB-EIA the optimal concentrations for HRP-conjugated protein G and sera were 1:60000 and 1:20 respectively. The maximum sensitivity and specificity were achieved when undiluted antigen (2 mg/mL) was used, higher dilutions of antigen resulted in reduced sensitivity of the test. In contrast, no significant staining was observed in the negative control (uninfected cells), even in the undiluted and different dilutions of positive and negative sera samples. In addition, no staining appeared when negative sera were applied to spotted antigen. We also observed that antigen spotted membranes are highly stable at -20 °C for up to 6 months, and it is not necessary to use freshly prepared antigen spotted membranes.

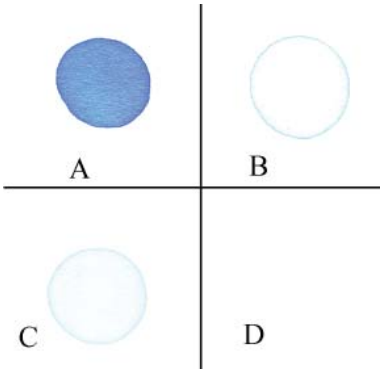

Fig.1. Examples of DB-EIA results: (A) undiluted positive serum with BVDV antigen, (B) undiluted positive serum with negative control antigen, (C) undiluted negative serum with BVDV antigen and (D) undiluted negative serum with negative control antigen

Table 1. Statistical analysis of SN and ELISA values scores from cattle sera tested for BVD antibodies

	SN+	SN-	
ELISA+	438	13	451
ELISA-	3	796	799
	441	809	1250

KAPPA= 0.972 (Martin et al., 1987)

Table 2. Statistical analysis of SN and DB-EIA values scores from cattle sera tested for BVD antibodies

	SN+	SN-	
DB-EIA+	433	36	469
DB-EIA-	8	773	781
	441	809	1250

KAPPA= 0.924 (Martin et al., 1987)

Table 3. Statistical analysis of DB-EIA and ELISA values scores from cattle sera tested for BVD antibodies

	ELISA+	ELISA-	
DB-EIA+	447	22	469
DB-EIA-	4	777	781
	451	799	1250

KAPPA= 0.955 (Martin et al., 1987)

Out of 1250 sera samples, 451, 441 and 469 were positive in ELISA, SN and DB-EIA tests, respectively. A total number of 431 samples were positive in all three tests. Chi-square statistical analysis showed a very highly significant association among these tests (Tables 1, 2 and 3).

Comparing all three tests, a higher number of positive sera were detected by DB-EIA test. Out of 469 sera detected positive by DB-EIA, 447 were detected positive by ELISA while only 433 were detected positive by SN test. Strong agreement (Kappa = 0.924) between the prevalence estimated by the DB-EIA and SN tests and also between ELISA and DB-EIA (Kappa= 0.955) was found when the population under study was analyzed as a whole.

Using SN test as a gold standard, the specificities of ELISA and SN test were as that of DB-EIA. The sensitivity and the specificity of ELISA were 99.31% and 98.39% respectively, whereas of the DB-EIA they were 98.18% and 95.6% respectively. The sensitivity and the specificity of the overlapped results with the DB-EIA and ELISA were 97.5% and 97.4%.

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 (LONERAGAN et al., 2005; ENOE et al., 2000). Evaluation of the DB-EIA in heat-inactivated sera (56 °C; 30 min) showed no difference between heated and unheated sera (results not shown). 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 ELSA tests, is that it does not detect antibody titer or optical density as a document.

In conclusion, the present study provides evidence that it is still possible to implement simple techniques for the sero-monitoring of BVD 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 pestiviral infections. To our best knowledge this is the first report of DB-EIA for the detection of BVD.

Acknowledgements

We gratefully acknowledge the assistance of Dr Hasan Momtaz, Dr. Ali Sharifzadeh from Azad University of Shahrekord and Dr. Masud Rohani, Veterinary Organization of Shahrekord, Iran for submitting cattle sera and technical assistance.

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Received: 7 April 2008
Accepted: 4 June 2009

HEMMATZADEH, F., F. AMINI: Dot-blot imunoenzimni test za dokaz protutijela za virus virusnoga proljeva goveda. Vet. arhiv 79, 343-350, 2009.

SAŽETAK

Dot-blot imunoenzimni test rabljen je za dokaz protutijela za virus virusnoga proljeva u zaraženih goveda. Kao antigen rabljen je virusni soj NADL. Ukupno je bilo pretraženo 1250 uzoraka seruma neutralizacijskim testom i imunoenzimnim testom. Protein G obilježen peroksidazom iz hrena rabljen je za dokaz specifičnih protutijela, a kao supstrat rabljen je TMB. Statističkom analizom nije ustanovljena značajna razlika u osjetljivosti dot-blot imunoenzimnoga testa, neutralizacijskoga testa i imunoenzimnoga testa. Razvijeni test je jednostavan, jeftin, pouzdan i brz te se može rabiti za serološku dijagnostiku virusnoga proljeva goveda.

Ključne riječi: dot-blot imunoenzimni test, virusni proljev goveda, serum-neutralizacijski test, imunoenzimni test