

## Dot-enzyme linked immunosorbent assay as an alternative technique for the detection of bovine respiratory syncytial virus (BRSV) antibodies

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**Abstract** – A dot-ELISA test for the detection of anti-BRSV antibodies is described. The objective of this study was the standardisation of a test as a fast, inexpensive and effective alternative to detect anti-BRSV antibodies. Its sensitivity, specificity and usefulness were compared to a commercial ELISA-kit and to the standard serum neutralisation (SN) test. The standardisation of the technique was done using nitrocellulose disks soaked with a viral sample isolated in Brazil, BRSV-25-BR. The best results were obtained when the disks were sensitised with a purified antigen at a concentration of 0.7 µg/disk and the bovine serum was diluted 1: 200. The experiment used 423 samples of bovine serum collected in the main cattle breeding centres in Brazil. The standard SN, dot-ELISA technique and commercial ELISA kits scored 67.8%, 71.8% and 72.3% of the samples as positive, respectively. When compared to the SN test, the standardised dot-ELISA and the commercial ELISA tests presented relative sensitivities of 92.3% and 91.6% and relative specificities of 71.3% and 68.4% respectively. The results demonstrated that the dot-ELISA test is adequate for the objectives proposed by this study, being easy to use and economically viable. Thus, this test represents an alternative for BRSV serological diagnosis in the substitution of SN and commercial ELISA tests, recommendable for utilisation in laboratories with few resources.

**bovine respiratory syncytial virus / dot-ELISA / commercial ELISA kit / serological analysis / Brazil**

**Résumé** – **Technique immunoenzymatique (dot-ELISA) pour détecter les anticorps contre le virus syncytial respiratoire bovin.** Cette étude décrit un test immunoenzymatique (dot-ELISA) pour la détection des anticorps contre le virus syncytial respiratoire bovin (anti-BRSV). Le but de ce travail est de standardiser une technique peu coûteuse et précise pour la détection des anticorps anti-BRSV. La sensibilité, la spécificité et l'utilité du test dot-ELISA ont été vérifiées et comparées à

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celles d'un kit commercial ELISA et du test de référence de séroneutralisation (SN). La standardisation de la technique a été réalisée sur disque de nitrocellulose imprégnée de l'échantillon BRSV-25-BR, isolé au Brésil. Les résultats ont été obtenus avec un disque sensibilisé par l'antigène purifié à la concentration de 0,7 µg de protéine purifiée et le sérum bovin à la dilution de 1 : 200. Des échantillons (423) de sérum obtenus dans les principaux centres d'élevage de bovins au Brésil ont été utilisés. Des résultats positifs ont été obtenus dans 67,8 % des échantillons par SN, 71,8 % par dot-ELISA et 72,3 % par le kit ELISA commercial. Par comparaison aux tests de SN, les tests dot-ELISA et kit ELISA commercial ont présenté des sensibilités relatives de 92,3 % et 91,6 % et des spécificités relatives de 71,3 % et 68,4 % respectivement. Les résultats montrent que le test dot-ELISA standardisé est approprié au but de cette étude, étant très facile à utiliser, économique et adaptable aux conditions brésiliennes. Ainsi ce test constitue une alternative aux tests de SN et ELISA commercial pour le diagnostic sérologique du BRSV. On peut donc conseiller l'utilisation de ce test pour le diagnostic de BRSV dans les laboratoires dépourvus d'appareils sophistiqués.

## **virus syncytial respiratoire bovin / dot-ELISA / ELISA commercial / test sérologique / Brésil**

### **1. INTRODUCTION**

Bovine Respiratory Syncytial Virus (BRSV) is an enveloped RNA virus that belongs to the Pneumovirus genus of the family Paramyxoviridae [1]. BRSV is one of the most important pathogens in bovine respiratory diseases and has a worldwide distribution, causing severe infections [1, 16, 22, 24].

Brazil is a great meat producer, with one of the largest commercial herds in the world. Very little is known about the significance of BRSV in Brazil. Recent studies have shown BRSV antibodies in numerous serum samples and BRSV specific antigens have been detected in histological specimens of bovines submitted to veterinary diagnosis in the southern and southeastern regions of Brazil [4, 9, 11, 13].

The importance to find and to standardise an efficient, fast and economically viable technique became apparent in order to assist the serological diagnosis of this disease whose significance has been increasingly recognised in our environment. Considering such observations, an alternative test, a dot-ELISA test, was developed in this study to detect the specific BRSV antibodies. Comparisons of the proposed technique with the commercial ELISA-kit

and the gold standard SNT (serum neutralisation test) are also provided in this work.

### **2. MATERIALS AND METHODS**

#### **2.1. Cells**

Continuous lineage Madin-Darby Bovine Kidney (MDBK) cells were cultivated in 75 cm<sup>2</sup> bottles (Corning, Inc. New York, NY, USA) with  $2 \times 10^5$  cells/mL (initial concentration) in minimum essential Eagle medium (MMEE-Cultilab, Campinas, SP, Brazil) with 10% fetal calf serum (FCS, Sigma Chemical Company, St. Louis, MO, USA).

#### **2.2. Virus**

The BRSV-25-BR strain isolated in Brazil was used for the development of dot-ELISA and SN. Bottles containing monolayers of MDBK cells, after a 24-hour incubation period, were inoculated with 0.01 to 0.1 Median Cell Culture Infective Doses (CCID<sub>50</sub>/mL) of the viral suspension for 1 hour at 37 °C, followed by the addition of MMEE. Approximately 5 to 7 days post-inoculation, when the monolayers presented an extensive cytopathic effect (CPE), that is, syncytial formation and cell

rounding [3], they were scraped and the cell lysate was clarified by centrifugation at  $1\,000 \times g$  for 10 min. The supernatant was stored at  $-70\text{ }^{\circ}\text{C}$  until use. For the preparation of antigen control, uninfected cells were processed in the same way as that described above.

### 2.3. Viral purification

The procedures for viral purification were those used by Gough and Collins [14], with some modifications. After BRSV replication in cell culture and clarification, the viral suspension was centrifuged at  $30\,000 \times g$  for 1 hour at  $4\text{ }^{\circ}\text{C}$  and subsequently the pellet was resuspended in TRIS-CaCl<sub>2</sub> (TRIS 1 M, CaCl<sub>2</sub> 1.5 mM, pH 7.4) buffer. The viral pellet was carefully added to a discontinuous sucrose gradient at two concentrations (30% and 55% v/v) and ultracentrifuged at  $53\,000 \times g$  for 1 hour 30 min at  $4\text{ }^{\circ}\text{C}$ . The fraction containing the virus at a concentration between 55% and 30% was harvested and centrifuged again at  $30\,000 \times g$  for 1 hour at  $4\text{ }^{\circ}\text{C}$ ; the pellet was resuspended in TRIS-CaCl<sub>2</sub> and stored at  $-70\text{ }^{\circ}\text{C}$  until use.

### 2.4. Determination of the infecting viral titers

The titrations were done on polystyrene 96-well microplates (Corning, Inc., New York, USA). Each well received 50  $\mu\text{L}$  of MMEE and 50  $\mu\text{L}$  of diluted viral suspension ( $10^{-1}$  to  $10^{-8}$ ), using 8 replicate wells per dilution. Thereafter, 100  $\mu\text{L}$  of a MDBK cell suspension ( $2 \times 10^5$  cells/mL) were added per well. Reaction-control wells received 100  $\mu\text{L}$  of MMEE and 100  $\mu\text{L}$  of cell suspension. The incubation took place in a humid atmosphere incubator of CO<sub>2</sub> at  $37\text{ }^{\circ}\text{C}$  (Forma Scientific, Mariette, Ohio, USA) for 5 days. The readings were taken by CPE in an inverted microscope (Carl Zeiss, Oberkochen, Germany). The titer (CCID<sub>50</sub>) was calculated by the Reed and Muench method [19].

### 2.5. Determination of protein concentration

The protein concentration of the samples was determined by the Lowry method [17], using bovine serum albumin as the standard sample (Sigma Chemical Company, St. Louis, MO, USA).

### 2.6. Serum samples

The serum samples were randomly collected from non-vaccinated animals in some of the main Brazilian centres of bovine production from 1998 to 2000. Altogether, 423 samples of bovine serum were collected in 6 different Brazilian states: Rio Grande do Sul (RS), Paraná (PR), São Paulo (SP), Mato Grosso do Sul (MS), Rio de Janeiro (RJ) and Minas Gerais (MG), as shown in Figure 1. A total of 21 different herds were investigated, involving 8 beef cattle herds (with 80 to 700 animals/farm) kept under extensive management and 13 dairy cattle herds (with 30 to 120 animals/farm). The samples, collected in all seasons from the animals of both sexes and different ages, originated from herds with a history of respiratory disease. The serum samples were centrifuged at  $1\,500 \times g$  for 10 min, inactivated ( $56\text{ }^{\circ}\text{C}$  for 30 min), aliquoted and stored at  $-20\text{ }^{\circ}\text{C}$  until use.

For all the serological tests described below, we also used BRSV positive ( $n = 10$ ) and negative ( $n = 10$ ) control sera produced by the Laboratório de Virologia Animal, Departamento de Microbiologia e Imunologia, Instituto de Biologia, Universidade Estadual de Campinas – UNICAMP, 13081-970 Campinas, SP, Brasil.

### 2.7. Serological methods

#### 2.7.1. Serum neutralisation test

The serum neutralisation test was conducted as described by Stephenson and Warnes [21] with pertinent modifications. Briefly, heat inactivated test sera were

mixed with approximately 100 CCID<sub>50</sub> of BRSV. After a one-hour incubation at 37 °C, 3 × 10<sup>4</sup> MDBK cells in 100 µL were added per well. The cells were subsequently incubated for five days and assessed microscopically. The titer was calculated by the Reed and Muench method [19].

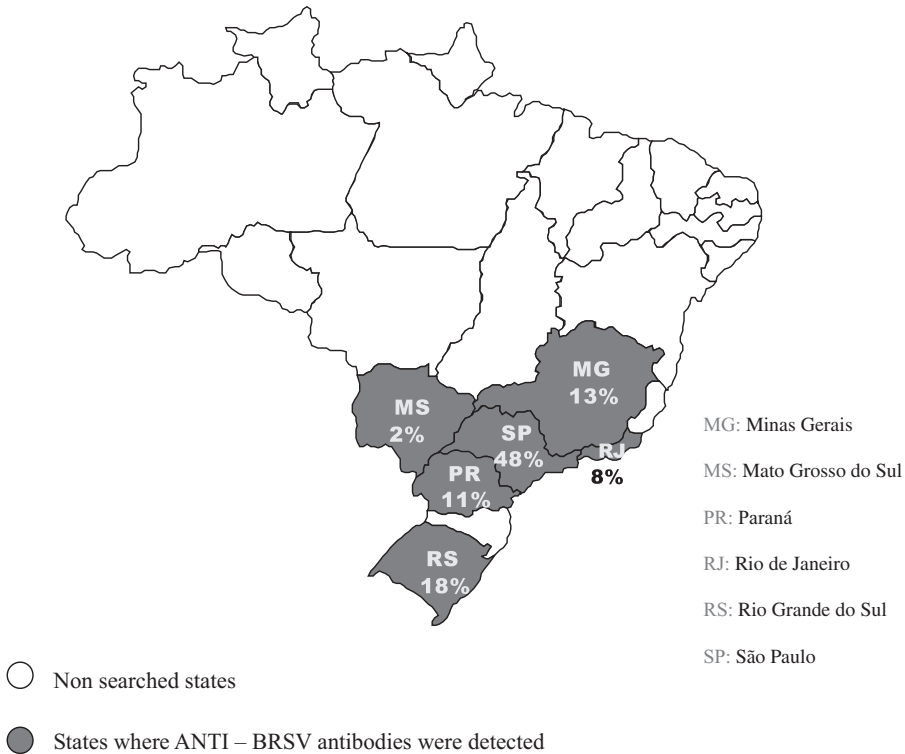
**2.7.2. ELISA kit**

The commercial ELISA-kit Svanovir™ (Svanova Biotech, Uppsala, Sweden) was compared with the dot-ELISA. The ELISA testing procedure and the interpretation of the results were performed according to the instructions of the manufacturer.

**2.7.3. dot-ELISA**

The purified virus antigen diluted in four different concentrations (1.0; 0.7; 0.4 and

0.2 µg/sheet) in carbonate-bicarbonate buffer (1.5 g Na<sub>2</sub>CO<sub>3</sub>, 2.93 g NaHCO<sub>3</sub>, 1 000 mL distilled H<sub>2</sub>O, pH 9.6) was spotted row wise onto the nitrocellulose membrane (NCM, HYBOND-C, NC, Amersham Biosciences Corp., Piscataway, NJ, USA) disks and incubated at room temperature for 30 min. After that, blocking was done with 1% skim milk in phosphate buffered saline (PBS) for 2 hours at room temperature. NCM disks were washed 4 times with PBS-T (8.0 g of NaCl, 0.2 g Kh<sub>2</sub> PO<sub>4</sub>, 1.28 g Na<sub>2</sub>HPO<sub>4</sub>.H<sub>2</sub>O, 0.2 g KCl and 0.5 mL Tween 20; pH 7.4). Next, 3 different dilutions (1:00, 1:200 and 1:400) of negative and positive sera, control and test sera were added and incubated at room temperature for 2 hours. The reactive samples were tested in duplicate. Subsequently, NCM disks were washed 4 times in PBS-T and incubated for 1 hour at room temperature



**Figure 1.** Proportion of Bovine Serum analyzed according to the State of Origin.

with 1:1000 diluted rabbit immunoglobulin anti-bovine IgG conjugated with peroxidase (Southern Biotechnology Associates, Inc. Birmingham, AL, USA) in PBS, following the manufacturer's recommendations. Then the NCM sheets were washed 4 times with PBS-T and treated with DAB (0.01% 3-3 diaminobenzidine tetrahydrochloride) in PBS containing 0.1% hydrogen peroxide at room temperature for 15 min. The addition of distilled water stopped the reaction and the results were read. The reactive samples were tested in triplicate. The results were scored as positive or negative after the visual observation of a colour reaction (dark, reddish grey) in the NCM disks as recommended by Cummins et al. [8].

## 2.8. Statistical analysis

A comparative study of the results achieved was done in the adapted dot-ELISA and ELISA Kit tests before the SN test, considering the SN test as standard proof. Relative sensitivity and specificity were evaluated as suggested by Coggon et al. [5]. In order to assess the level of agreement between dot-ELISA and commercial ELISA Kit tests, the index of concordance, Kappa ( $\kappa$ ), was used [20, 23].

## 3. RESULTS

### 3.1. Standardisation of the dot-ELISA

The immunoenzymatic technique called dot-ELISA was adapted and standardised

for detection of anti-BRSV antibodies. In the viral titration and protein concentration dosage tests, the value obtained was  $10^{5.7}$  CCID<sub>50</sub>/mL and 970 mg/mL for purified antigen samples. The optimal concentrations of the antigen and serum for the dot-ELISA test were determined by a checker board, using different concentrations of antigens and control sera. The antigen concentration of 0.7  $\mu$ g/disk and diluted serums of 1:200 resulted in a major difference between the positive and negative results. All reactions were tested two-fold, with positive and negative control sera, so that it would be possible to compare each sensitised disk plate.

### 3.2. Serological analysis

Seroreactors were found in all the states studied (Fig. 1). Amongst the 423 serum samples analysed, 67.8%, 71.8% and 72.3% were positive for the presence of BRSV antibodies in the SN, dot-ELISA and ELISA kit tests, respectively (Tabs. I and II). The standardised (dot-ELISA) and the commercial ELISA tests presented a relative sensitivity of 92.3% and 91.6% and a relative specificity of 71.3% and 68.4% respectively, as compared to the SN tests (Tab. III). According to the adopted statistical analysis, these relative values were not significantly different.

## 4. DISCUSSION

In this paper, we propose a fast and non-expensive standardised dot-ELISA

**Table I.** Comparison of serological examinations of antibodies against BRSV using SN and dot-ELISA.

	SN Positive	SN Negative	Total
Dot-ELISA Positive	265	39	<b>304 (71.8%)</b>
Dot-ELISA Negative	22	97	<b>119 (28.1%)</b>
<b>Total</b>	<b>287 (67.8%)</b>	<b>136 (32.1%)</b>	<b>423</b>

**Table II.** Comparison of serological examinations of antibodies against BRSV using SN and commercial ELISA-kit.

	SN Positive	SN Negative	Total
<b>ELISA-kit Positive</b>	263	43	<b>306 (72.3%)</b>
<b>ELISA-kit Negative</b>	24	93	<b>117 (27.7%)</b>
<b>Total</b>	<b>287 (67.8%)</b>	<b>136 (32.1%)</b>	<b>423</b>

**Table III.** General Evaluation of the Tests Developed according to the Serum Neutralisation test.

Characteristic	dot-ELISA	ELISA-Kit
Sensitivity (%)	92.3	91.6
Specificity (%)	71.3	68.4
Positive Predictive Value (%)	87.2	85.9
Negative Predictive Value (%)	81.5	79.5
Global Performance (%)	85.5	84.2
Kappa	0.658	0.623
P Kappa Value	< 0.005	< 0.005

test designed to detect BSRV. The test was applied to samples collected in main cattle breeding centres in Brazil and the results were compared with corresponding applications to SN (Tab. I).

For dot-ELISA assays, several parameters were evaluated. These included the use of different concentrations of antigen, serum samples and conjugated, with a particular attention to the involved costs (materials and processing effort). The optimal reaction concentrations were determined in order to obtain the best discrimination between the reference positive and negative sera. Grover and Guillemain [15] suggest that the optimum reagent times and dilutions of the different phases of the technique should be standardised, since the detected antibodies and detection time depend on the technique chosen.

The aim of our study was to evaluate the performance of the dot-ELISA by compar-

ing it to the available serological tests (Tabs. I and II). The serum samples were randomly collected and as a consequence, a detailed serological analysis was considered out of scope and not carried out. Moreover, the great discrepancy between the number of samples originating from the different regions studied (Fig. 1), did not allow any regional statistical evaluation.

Amongst the 423 serum samples analysed, 67.8%; 71.8% and 72.3% were positive for the presence of BRSV antibodies in the SN, dot-ELISA and commercial ELISA kit tests, respectively (Tabs. I and II). The high proportion of herds with the BRSV antibody agrees with the results reported in Brazil [4], the United States [6], England [18], Canada [25], and, more recently, Uruguay [7].

The commercial kit ELISA Svanovir™ (Svanova Biotech, Uppsala, Sweden) proved to be efficient in the detection of anti-BRSV

antibodies. However, some disadvantages should be considered, such as the need to use an absorbance reader, which is expensive and therefore difficult to obtain for laboratories with limited resources, as well as the high cost of the kits. The results obtained with the dot-ELISA test presented a satisfactory degree of concordance with the commercial kit used and with the gold standard SN test, which has several inherent limitations, such as the complexity involved in cell cultivation and being laborious and time demanding [10, 12, 26]. The dot-ELISA proved to be a quick test, easy to be performed in relatively well-equipped laboratories. Besides these advantages, the studies carried out by Woodruff et al. [27] and Bosompem et al. [2] indicate that the NC disks impregnated with the antigens could be perfectly stored at 4 °C without efficiency loss.

In conclusion, the performance characteristic of the dot-ELISA adapted in this study for the detection of anti-BRSV antibodies represents a competent substitute for the commercial ELISA and the SN test in relatively well-equipped laboratories.

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