The development and application of an indirect ELISA test for the detection of antibodies to bovine respiratory syncytial virus in blood serum

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ABSTRACT: We developed an indirect enzyme-linked immunosorbent assay (ELISA) for detection of serum antibodies to bovine respiratory syncytial virus. For evaluation of the newly developed ELISA, field sera collected from 549 head of cattle in the Czech Republic were tested in parallel by a serum neutralization test. The tests showed 98.36% agreement. The specificity and sensitivity of the ELISA relative to serum neutralization test was 97.00% (226/233) and 99.37% (314/316), respectively. Tissue culture-grown viral antigen was used in the tests. The corrected optical density (COD) of each sample tested at dilution 1/100 was expressed as a percentage of the COD of a positive reference serum included on each plate, this value was the sample/positive (S/P) ratio. We determined the relationship between the S/P ratio (%) obtained at a dilution 1/100 and the end point titer calculated by serum neutralization test ($r = 0.9743$). The ELISA test was evaluated by testing acute and convalescent (3 wk later) serum pairs from 9 head of cattle with confirmed BRSV infection for demonstration of seroconversion. The ELISA test demonstrated a clear increase of the S/P ratio (%) between acute and convalescent serum pairs (on average 42.2 ± 13.1).

Keywords: bovine respiratory syncytial virus (BRSV); respiratory tract pathogen; cattle; ELISA

A list of abbreviations: BRSV – bovine respiratory syncytial virus, SN test – serum neutralization test, HIT – haemagglutination inhibition test, FAT – fluorescent antibody test, CPE – cytopathic effect, OD – optical density, PBS-T – phosphate buffered saline with Tween 20, MEM – minimal essential medium, RT-PCR – reverse transcription-polymerase chain reaction.

INTRODUCTION

A complex of mass diseases of the respiratory system referred to as respiratory syndrome is the cause of considerable economic losses in calf and young cattle rearing. The Bovine Respiratory Syncytial Virus (BRSV) is one of the most important and, in the Czech Republic, somewhat underestimated causes of the respiratory syndrome in calves and young cattle.

BRSV was first isolated in 1967 in Switzerland (Paccaud and Jaquier, 1970) from calves with a respiratory tract disease. In the Czech Republic, the virus was successfully isolated in 1976 from an acute outbreak of respiratory disease in feedlot cattle (Pospíšil *et al.*, 1978). Both the isolation of the virus and serological studies suggest that BRSV causes outbreaks of acute respiratory diseases worldwide (Edington and Jacobs, 1970; Wellemans *et al.*, 1970; Jacobs and Edington, 1971; Köves and Bartha, 1975).

BRSV is unusually sensitive to the outer environment and its isolation from clinical specimens is very exceptional and therefore is not recommended as a routine method. The most frequent diagnostic methods are serological methods – serum neutralization test (Baker *et al.*, 1986), complement fixation test, indirect fluorescent antibody test and ELISA test (Gillette, 1983; Elvander *et al.*, 1995). BRSV was isolated in a cattle herd in the Czech Republic in 1996 from an acute outbreak of respiratory disease (Kovařčík, 1999). Between 1998, and 1999 the BRSV infection was also serologically diagnosed (SN test) in other herds affected by serious respiratory disease. Despite efforts to perfect micromethods, SN test remains a time-consuming technique with difficult result evaluation and is therefore used rather in research studies than in routine diagnostics. This is why the indirect ELISA test was developed for the detection of antibodies to BRSV in blood serum. This test simplifies the diagnostics of BRSV infections and can be used as a routine diagnostic method.

This article describes the development and use of the ELISA test for demonstrating BRSV anibodies and compares the specificity and sensitivity of the newly developed ELISA test with a SN test.

MATERIAL AND METHODS

Serum samples

A total of 549 blood serum samples from 16 herds in the Czech Republic were examined. The field sera collected from animals of all age groups from 14 non-BRSV-vaccinated herds and 2 herds vaccinated with inactivated BRSV vaccine were tested parallelly by ELISA and SN test. The sera were stored at –20°C after being inactivated for 30 minutes at 56°C.

Next, 9 blood serum samples were examined from a group of animals in which infection by the BRSV virus was detected through isolation on cell cultures or through direct fluorescent antibody test (FAT). Serum samples were taken from animals of two herds (serial number 1 to 7 and 8 to 9) in the acute phase of the disease and after 3 weeks in the convalescent phase of BRSV infection (paired serum samples).

Virus and cell cultures

BRSV strain VS 97 (catalogue number CAMP V-534, Collection of Zoopathogenic Microorganisms, VRI Brno, Czech Republic) was propagated on a stable cell line MDBK (catalogue number 90050801, European Collection of Animal Cell Cultures, United Kingdom). Viral suspension (1 000 $TKID_{50}$/0.1 ml) was absorbed into a monolayer of cells for 1 hour at 37°C. Cells were cultivated in Roux 1 200 bottles (Simax, Czechoslovakia) and kept in 100 ml of minimal essential medium Eagle (catalogue number M-0643, Sigma), which was supplemented with a 2% pre-colostral serum (SML-ZVOS Hustopeče Ltd., Czech Republic). Parallelly to the infected cells, uninfected cells were cultivated for the preparation of negative antigen. Cells were incubated at 37°C.

Preparation of BRSV antigens for the ELISA test

When the cytopathic effect was at its peak, reaching 90 to 100% cells, the remaining cells were mechanically shaken off onto the medium. These cells were centrifuged at 2 000 × g for 10 minutes. The pellet was re-suspended in a small amount (100 times smaller than the amount of the medium) of 0.5% Igepal CA – 630 solution (catalogue number I-3021, Sigma) in PBS 7.2 and incubated at 4°C for 1 hour. The detritus of cells was removed by centrifugation at 2 000 x g for 20 minutes. Control antigen was prepared in parallel from uninfected cells.

The ELISA test procedure

One hundred microliters of the BRSV and control antigen, in an appropriate dilution ranging between 1 : 200 and 1 : 1000 in 0.05 M carbonate-bicarbonate buffer (pH 9.6), were dispensed into a 96-well microtiter plates (Nunc). The plates were incubated overnight (> 16 hours) at 4°C, washed in PBS-T three times and kept at 4°C.

The tested sera were diluted at 1 : 100 in PBS-T and added into wells containing positive and negative antigens in 100 µl doses. According to the recommendations by the Joint FAO/IAEA Division, a negative, a weak positive and a strong positive control were run as standards in duplicate wells on each microtiter plate.

The plates were sealed and incubated for 1 hour at 37°C. After each incubation step in the ELISA procedure, nonbound material was removed after washing 3 times in PBS-T. Then 100 µl of conjugate (Dalko P 0159) diluted according to the manufactur's instructions at 1 : 5 000, was added to each well and ancubated for 1 hour at 37°C. After washing, 100 µl of freshly prepared TMB substrate (0.1 mg/ml tetramethylbenzinide dissolved by dimethylsulfoxide in 0.1 M sodium acetate buffer [pH 6.0] cotaining 0.04 µl of 30% H_2O_2) was added o each well. The reaction was stopped using 100 µl of 1 M H_2SO_4 after 10 minutes at room temperature.

The results were established by calculating the final optical density (OD) of the examined sample from the difference between the OD of BRSV antigen and the OD of negative antigen. The sera samples were evaluated as positive if their pure optical density was higher than 0.2. This value was established by adding the average OD_{450} and the standard deviation multiplied by 3. The values of the average OD_{450} (0.041) and the standard deviation (0.052395) were established from a set of 50 blood serum samples from the BRSV-free animals (Cremer *et al.*, 1982; Elvander *et al.*, 1995).

Definition of positive standards in ELISA test

A positive standard was chosen from collected sera with serum-neutralizing antibodies (at least 1 : 64). Pooled sera were selected according to the recommendation of FAO/IAEA (Report of a joint FAO/IAEA Consultants Meeting on ELISA Data Evaluation and Expression, January 27–31, 1992).

Definition of negative standards in ELISA test

A negative standard was chosen from collected sera which had been negatively tested for the presence of serum-neutralizing antibodies (less than 1: 2) to the BRSV virus according to the recommendation of FAO/IAEA.

Serum neutralization test was carried out by a micromethod in plates (GAMA Ltd., České Budějovice, Czech Republic). The serum samples which had been diluted at 1 : 2 to 1 : 256 (two-fold dilution), were incubated in the volume of 50 µl with the same volume of the viral

suspension containing approximately 100 $TKID_{50}$/0.1 ml of BRSV. After incubating for 1.5 hours at room temperature, a suspension of secondary fetal calf kidney cells in MEM Eagle with 30% of precolostral bovine serum was added in the volume of 50 ml (5×10^5 cells/ml). The plates were incubated at 37°C in 5% CO_2 humidified atmosphere for 5 to 6 days. Serum titres were established as reciprocal values of the highest serum dilution which completely inhibit CPE in at least one out of two wells.

Detection of antibodies to other bovine viruses

Commercial ELISA tests have been used to detect antibodies to the infectious bovine rhinotracheitis (IBR) and bovine virus diarrhoea virus (BVDV) according to the manufacturer's instructions (Test-Line Ltd., Clinical Diagnostics, Brno, Czech Republic). A standard haemagglutination-inhibition test (HIT) was used to detect antibodies to the parainfluenza virus 3 (PI-3).

Haemagglutination-inhibition test

HIT for the detection of antibodies to the PI-3 virus was carried out in microtiter plates type U (GAMA Ltd., České Budějovice, Czech Republic). All the test components were measured in 50 ml doses and diluted in PBS 7.2. The serum samples were diluted at 1 : 2 to 1 : 256 (two-fold dilution) in a total of 3 columns. The PI-3 virus was then added to the first two columns in amounts of 4 haemagglutination units. PBS was added to the third column in place of the virus. After the incubation period of 1 hour at room temperature, 0.7% suspension of guinea-pig erythrocytes was added and the plate was thoroughly shaken. Each serum was examined in three columns, where the first two columns contained a mixture of serum and antigen for the detection of specific haemagglutination inhibition and the third column contained a mixture of serum and PBS as a control of nonspecific agglutination of the serum examined.

The results were assessed following the sedimentation of control erythrocytes. The serum titers were established as reciprocal values of the highest dilution of the sera which inhibited haemagglutination in both wells.

Immunofluorescent test

We used a direct fluorescent antibody test to detect BRSV with the aid of conjugate FITC-Ig against BRSV (CVL Weybridge, United Kingdom, catalogue number PA 0204) in cellular detritus obtained from nasal secretion. Nasal swabs were taken from the caudal part of the nasal cavity. After the nasal swabs were taken, the swabs were inserted into a test tube containing 0.5 ml of PBS (pH 7.6)

and soaked for 1 hour at 4°C while being shaken thoroughly from time to time. The PBS including the cellular detritus was then centrifuged at 14 000 rpm for 10 seconds in an Eppendorf centrifuge. The pellet was re-suspended in 50 µl of PBS (pH 7.6) and transferred onto a slide. After air drying, the cellular detritus was fixed in cooled acetone for 10 minutes. After washing in PBS for 10 minutes, conjugate was added in a 1 : 20 dilution (manufacturer's recommendation). Incubation took place in a moist chamber at 37°C for 30 minutes. Following the incubation, the slide was rinsed in PBS three times for 5 minutes. A PBS/glycerol mixture was transferred onto plates with the cellular detritus and it was then covered with a cover-glass.

Results were read with the help of a fluorescent microscope.

Isolation and identification of the virus

The isolation and identification of the virus were carried out according to a previously described method (Kovařčík, 1999).

Statistical analysis

As the data we obtained corresponded to the condition of normality, it was possible to make use of parametric tests in the statistical assessment. Correlative analysis was used to establish dependency and the analytical error was calculated as a standard deviation of the differences in paired values (119 duplicates of serum samples).

S/P ratios were calculated according to the following equation: S/P ratio (%) = 100 × [corrected optical density of a sample/corrected optical density of a positive reference serum]. Antibody activity of the samples examined in the indirect ELISA test is, by means of S/P ratios, expressed as a percentage of positivity of strong positive standard.

The analytical error of the method was calculated according to the following formula:

$$S = \sqrt{\frac{\sum d^2}{2n}}$$

where:

n = the number of the duplicates examined,

d = the difference between corrected absorbances (or S/P ratios) of twice examined same serum samples.

Sera duplicates were examined both from the perspective of intra-assay and from the perspective of inter-assay.

RESULTS

Comparison between ELISA and SN test

In total, 549 blood serum samples were examined in parallel by both ELISA and SN tests. When comparing the tests, 314 samples were positive and 226 negative in both cases, which represents a consensus in 98.36% of samples. Differences were found in a total of 9 samples (Table 1).

Table 1. Determination of sensitivity and specificity of the indirect ELISA test for the detection of antibodies to bovine respiratory syncytial virus in comparison with serum neutralizing test

	SN test		
	positive	negative	total
ELISA			
positive	314	7	321
negative	2	226	228
Total	316	233	549

Two samples which were positive in SN test were negative in the ELISA test (absorbing values: 0.168; 0.134), which corresponds to 99.37% of the ELISA test sensitivity in comparison with SN test.

Seven samples which were negative in SN test were positive in ELISA (absorbing values: 0.209; 0.235; 0.330; 0.269; 0.339; 0.292; 0.216) which corresponds to 97.00% of the ELISA test specificity in comparison with SN test.

Determining BRSV antibodies of paired blood serum samples in a group of animals with confirmed BRSV infection by FAT or isolation

Nine serum samples were examined from animals which were in the acute phase of the disease and nine serum samples from animals in the convalescent phase (after 3 weeks) of the BRSV infection. Only 1 serum sample which had been collected in the acute phase of the disease was positive. All serum samples which had been collected in the convalescent phase of the disease were positive (Table 2). The increase of S/P ratios (%) in the convalescent sera amounted to 42.2 ± 13.1 (average ± standard deviation).

Determining antibodies to other respiratory viruses

All nine cows were examined for the presence of antibodies to the BVD, IBR and PI-3 viruses. In the acute phase of the disease, one serum sample was positive for the antibodies to the BVD virus and all nine samples were positive for the antibodies to the PI-3 and IBR viruses. The convalescent serum samples had the same results as the sera in the acute phase of the disease and no active seroconversion (an increase of the specific antibody level) against the BVD, IBR and PI-3 viruses was observed.

Table 2. Detection of BRSV antibodies using the newly developed ELISA test in paired samples of blood sera collected in the acute (A) and convalescent (B) phases of the disease

Animal No.		ELISA		Virus isolation	FAT
		OD	S/P (%)		
1	A	0.061 –	6.3	–	+
	B	0.722 +	74.4		
2	A	0.373 +	38.5	–	+
	B	0.572 +	59. 0		
3	A	0.032 –	3.3	–	+
	B	0.376 +	38.8		
4	A	0.156 –	16.1	–	+
	B	0.441 +	45.5		
5	A	0.021 –	2.2	–	+
	B	0. 457 +	47.1		
6	A	0.068 –	7. 0	–	+
	B	0.500 +	51.5		
7	A	0.030 –	3.1	–	+
	B	0.416 +	42.9		
8	A	0.063 –	4.6	+	not tested
	B	0.635 +	46.6		
9	A	0.074 –	5.4	+	not tested
	B	0.829+	60.8		
		*42.2 ± 13.1			

*average ± standard deviation

Establishing a dependency between a SN titre and S/P ratio set by the ELISA test

The dependency between the SN titres and S/P ratios set by the ELISA test in 43 blood serum samples is indicated on the graph by a regressive straight line and correlation coefficient r (Figure 1). We found a high correlation coefficient ($r = 0.9743$), suggesting a strong relation between the SN titre and S/P ratio set by the ELISA test.
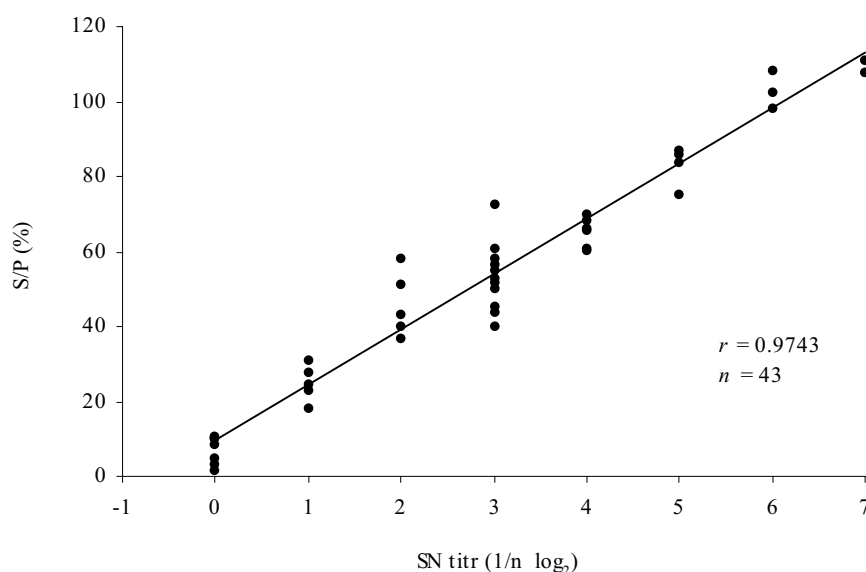
Figure 1. Dependency of SN titres and S/P ratios determined by the ELISA test

This correlation coefficient is of high statistical significance ($P < 0.01$). The graphic demonstration of the regressive straight line and the calculation of the correlation coefficient were both achieved using the Microsoft® Excel 97 programme.

Analytical error of the ELISA method

The analytical error of the intra-assay ELISA test expressed in percent according to the formula: $100 \times$ [analytical error of method (S)/mean value of absorbance (alternatively of S/P ratio)] comes to 8.1%. The analytical error of the inter-assay ELISA method expressed in percent according to the same formula is 19.7% for the absorbance and 14.3% for the S/P ratio.

The analytical error of the method was established from 119 duplicates of serum samples.

DISCUSSION

Serological methods belong among the most frequently used diagnostic methods for infections caused by BRSV because a direct virus isolation on cellular cultures is difficult and time-consuming. The ELISA test is generally considered to be a specific and sensitive method for detecting serum antibodies to BRSV in comparison with the precise and sensitive serum-neutralizing test.

Our results obtained by comparing the ELISA test and SN test demonstrated 99.37% sensitivity, where 2 samples which were positive in SN test were negative in the ELISA test (Table 1). The specificity came to 97.00% as 7 samples were negative in SN test but positive in the ELISA test. Absorbance values of these serum samples were low (0.209–0.339). There are two possible explanations for the variance in the results of the 9 samples. One of

the possible causes of such variance could be an analytical error of the method. The other cause could be diversity in the concentration of neutralizing and total BRSV-reactive antibodies in naturally infected cattle and following the administration of inactivated vaccines of various origin (Ellis *et al.*, 1995). The ELISA test detects total BRSV-reactive antibodies (including those which are incapable of neutralization) whereas SN test only detects neutralizing antibodies. Three questionable samples originated from one herd of adult animals between the ages of 18 to 36 months where an inactivated BRSV vaccine was used.

In order to be able to use a serological method in practice, it is essential to detect changes in antibody titers following an infection caused by BRSV. Some studies state that the ELISA test failed in demonstrating seroconversion and SN test proved to be the most effective (Gillette, 1983). The cause of the ELISA test failure in demonstrating seroconversion following an experimental infection may be the use of class-specific (anti-IgG) conjugate which is incapable of detecting class IgM antibodies. After experimentally infecting non-colostral calves, IgM-class antibodies were detected 8 to 10 days post infection (PI), while IgG1-class antibodies were detected 13 to 17 days PI (Kimman *et al.*, 1987). Similar results were obtained in another experiment where IgG1-class antibodies were detected 10 to 16 days PI. IgM-class antibodies were detected 10 days PI, they reached their peak on the 16th day PI and after that their level fell sharply until the 38th day after infetion when they were undetectable (Florent and Wiseman, 1990). In both experiments IgM and IgG1 class antibodies were detected at approximately the same time. Titers of IgG1 antibodies were very low for one week, while titers of IgM antibodies quickly increased. For this reason the results obtained through the ELISA test using class-specific conjugates (anti-IgG) in combination with a low number of samples need not

lead to the unambiguous establishment of seroconversion. In the ELISA test we used rabbit conjugate against bovine immunoglobulins (both IgG and IgM), by which means we gained relatively unambiguous results in demonstrating seroconversion following natural infection (Table 2). The comparison of paired samples of blood sera may be carried out within the framework of one test (intra-assay) using the corrected optical density of the samples. Within the framework of two tests separated in time (interassay) it is more appropriate to compare the use of S/P ratios, which has the advantage of a lower analytical error of the method. These indirect methods of diagnosing infection may be supplemented by direct methods. Given that the isolation of BRSV is difficult and time-consuming, it is not recommended as a routine method. For this reason it is often replaced by methods of direct immunofluorescence with monospecific serum in nasopharyngeal samples (McNulty *et al.*, 1983) or in sections of lung fragments, especially from the apical and cardial lobes (Thomas and Stott, 1981). Recently new diagnostic methods for detecting BRSV have also been developed, for example RT-PCR (Vilcek *et al.*, 1994; Henkel *et al.*, 1997).

The indirect ELISA test is a a fast and reliable method for detecting BRSV antibodies in blood serum, permitting its routine use in the diagnosis of infections caused by BRSV.

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