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1989

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Rhodes, Marvin B.; Klucas, Carol A.; Frey, Merwin L.; and Anderson, Gary A., "A blocking ELISA for the detection of specific antibodies to bovine respiratory syncytial virus" (1989). *Roman L. Hruska U.S. Meat Animal Research Center*. 438.  
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## A blocking ELISA for the detection of specific antibodies to bovine respiratory syncytial virus

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**Abstract.** A blocking enzyme-linked immunosorbent assay (ELISA) has been adapted to detect specific antibodies in bovine sera to respiratory syncytial virus using a horseradish peroxidase-labeled monoclonal antibody to the fusion protein of the virus. This assay plus an indirect blocking ELISA and indirect ELISA were used to detect antibodies to the bovine respiratory syncytial virus (BRSV) in 159 field-origin bovine sera. Results of these assays were compared with serum antibody titers measured by the serum neutralization (SN) test. Over a 56-day period, the mean neutralization titers and the mean delta absorbance values for the blocking ELISA, on the same sera, showed similar declines. However, the calculated correlation coefficients between mean SN titer and mean absorbance value for the blocking ELISA of the individual sera ranged from -0.2 to -0.5 depending on the source of sera. Similar values were obtained whether using crude or purified viral antigen in the assays. Corresponding calculated correlation coefficients were generally higher for the indirect blocking ELISA or indirect ELISA than for the blocking ELISA. The blocking ELISA was between 70 and 64% as sensitive as the serum neutralization test with a specificity of 100 or 90% using the crude and purified viral antigen, respectively. The indirect blocking ELISA and indirect ELISA had similar calculated sensitivities and specificities. The blocking ELISA was faster to run than either of the other ELISA's or the neutralization test. Further, nonspecific background absorbance was obviated because the blocking ELISA detects antibodies to 1 specific viral protein, the fusion protein. These studies suggest that the blocking ELISA should be useful as a serological test for BRSV antibodies.

Bovine respiratory syncytial virus (BRSV) is an important cause of respiratory disease in cattle. Serological methods currently in use include the serum neutralization (SN) test,<sup>2,4,10,14</sup> complement fixation test,<sup>5</sup> indirect fluorescent antibody test,<sup>8</sup> and the enzyme-linked immunosorbent assay (ELISA).<sup>4,8</sup> An antibody-capture ELISA has been introduced that distinguishes between BRSV-specific IgM and IgG.<sup>15</sup> In a study<sup>5</sup> comparing the SN test and ELISA titers, a correlation ( $r = 0.75$ ) was obtained between the 2 tests on sera from experimentally infected calves, but a correlation of 0.25 was obtained between these assay results for sera from feedlot calves. Perhaps the lower correlation may have been due to high nonspecific background associated with some field sera.<sup>4</sup>

A competitive ELISA has been described for the detection of antibodies to bovine herpesvirus-1.<sup>3</sup> The purposes of this study were to adapt this competitive ELISA (a type of blocking ELISA) for detection of antibodies to BRSV and to compare the results obtained with the SN test. It was postulated that non-

specific background would thus be obviated by the blocking ELISA compared to the indirect ELISA.

### Materials and methods

**Monoclonal antibodies (Mab's).** The MAb's to the fusion protein of BRSV were prepared as described? Monoclonal antibody 8G12 was purified from ascites fluid by precipitation with a final concentration of 2.05 M ammonium sulfate (50% saturation), pH 7.0, and labeled with horseradish peroxidase<sup>a</sup> (HRP0).

**Viral antigens.** A crude viral antigen was prepared from BRSV-infected bovine turbinate cell cultures, which were harvested at 85% cytopathic effect and stored at -60 C. The cell culture material was thawed and centrifuged at 82,500 x g<sup>b</sup> for 1 hr. The supernatant fluid was discarded and the pellet resuspended in phosphate-buffered saline (PBS) and sonicated<sup>c</sup> for 10 sec. This crude viral antigen was stored at -20 C. An aliquot of the crude antigen was treated with 0.1% Nonidet P-40 at 37 C for 17 hr. The solution was fractionated on a 10-40% (w/w) sucrose density gradient by centrifuging at 82,500 x g for radioimmunoassay (RIA) (Rhodes MB, Klucas CA, New CW Jr, Anderson GA: 1986, Abstr Conf Res Workers Anim Dis # 145). Three 1-ml fractions from the top of the gradient contained the viral antigens and were combined. This material was stored at 20 C and is referred to as the purified viral antigen.

**General procedures for the ELISA's.** Buffers and reagents used for the ELISA procedures have been described.<sup>12</sup> The 3 types of ELISA used in this study were similar procedurally

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Presented at the 31st Annual Meeting of the AAVLD, Little Rock, AR, October 15-18, 1988.

Received for publication October 14, 1988.

for the first steps. Carbonate buffer (0.05 M, pH 10) was added to the wells of a polystyrene plate<sup>d</sup> and allowed to stand overnight at 4 C and then removed. Crude (1.28 µg/well) or purified viral (0.12 µg/well) antigens diluted in carbonate buffer were added in 50-µl aliquots to the wells of the plate and dried with a 40 C stream of air for 30 min. The plates were stored at 4 C or used directly for 1 of the ELISA procedures or for the IRIA. Before use, the plate with the dried antigen was washed 2 times with ELISA wash solution and once with distilled water. Bovine serum albumin<sup>a</sup> (100 µl of a 1% solution in carbonate buffer per well) was added to block sites in the well not occupied by the viral antigen. The plate was incubated at 37 C for 30 min, the solution was removed and the plate was then ready for the individual ELISA procedures. All sera used in this study were diluted in conjugate serum diluent 1:40 and applied to duplicate wells in the plate. Known positive and negative sera were included on each plate.

**Specific procedures for ELISA's.** Brief outlines of the 3 ELISA's used are as follows:

Blocking ELISA: 50 µl serum, 30 min, 37 C, plus 50 µl HRPO-anti-fusion protein MAb, 30 min, 37 C; wash 3 x (as above); 100 µl substrate, 30-60 min, 25 C; read on ELISA Reader.<sup>e</sup>

Indirect blocking ELISA: 50 µl MAb 8G12 (1:10,000), 30 min, 37 C, plus 50 µl serum, 30 min, 37 C; wash 3 x; plus 50 µl HRPO-anti-bovine IgG<sup>f</sup> (1:500), 60 min, 37 C; wash 3 x; 100 µl substrate, 30-60 min, 25 C; read on ELISA Reader.

Indirect ELISA: 50 µl serum, 120 min, 37 C; wash 3 x; 100 µl HRPO-anti-bovine IgG (1:500), 120 min, 37 C; wash 3 x; 100 µl substrate, 30-60 min, 25 C; read on ELISA Reader.

Checkerboard titrations of the antigens and conjugates were done to determine optimal conditions for the blocking ELISA. Optimal crude and purified viral antigen concentrations, determined by this titration, were used in titrating sera and conjugates used in the indirect blocking ELISA and indirect ELISA. Unlabeled MAb 8G12 was titrated using the blocking ELISA and diluted so that it blocked 90% of the absorbance of the HRPO-anti-fusion MAb. The IRIA was done as previously described<sup>9</sup> except <sup>125</sup>I-labeled anti-bovine IgG was prepared and used as the detector.

**Expression of results.** In the blocking ELISA, the delta absorbance equals the absorbance with the conjugate on the viral antigen alone minus the absorbance with the serum and the conjugate. Sera were considered positive for antibodies to BRSV if the delta value was greater than 10% of the absorbance with the conjugate alone. For the indirect blocking ELISA and indirect ELISA, the delta absorbance equals the absorbance for an unknown serum minus the absorbance for the negative sera. Sera were considered positive for antibodies to BRSV if the delta value was greater than the mean absorbance for the negative sera plus 2 standard deviations of this value. A known positive antiserum to BRSV (78-16, SN titer = 1:640) and a known negative serum (78-17, SN titer = < 1:5) were included on all plates. Sera with a SN titer of < 1:5 were considered negative by this test.

**Source of sera.** The sera from Group A animals were from 43 calves born in late February, March, April, and early May.

There were multiple bleedings (2-4 per calf) included for 29 of 43 calves between November 11 and January 9. Calves were vaccinated against bovine viral diarrhea, parainfluenza-3, and bovine herpesvirus-1 viruses when they were put in the feedlot (October 11). Twenty-two of the 43 calves also were vaccinated with BRSV. Sixty-seven sera (Group B) were obtained from the Veterinary Diagnostic Center, University of Nebraska-Lincoln, Lincoln, Nebraska. All sera were assayed by the SN test<sup>1</sup> and the above-described ELISA's.

**Calculations.** Data were expressed as coefficients of correlation (*r*)<sup>11</sup> between SN titers and delta absorbance for the ELISA's. Sensitivity and specificity values<sup>13</sup> were calculated using the number of positive and negative sera as determined by the SN test to compare with the number of positive and negative sera by the ELISA procedures.

## Results

Results of Nonidet P-40-treated BRSV fractionation by sucrose density centrifugation shows that the viral antigens were present primarily in the top 3 fractions, as detected using serum 76-16 or MAb 8G12 in an IRIA (Fig. 1). However, field bovine sera 0044 and 2806 (Group A) appear to contain antibodies to antigens throughout the gradient (Fig. 2). Some sera reacted more strongly with the antigens in fractions 4-12 than others. Sera from each of the 2 calves gave similar delta cpm for reaction with antigens in fractions 1-3 for each of the 2 serum samples regardless of the SN titers of the sera.

The geometric mean of the SN titers was plotted according to bleeding dates for the calves in Group A (Fig. 3). Similarly, mean delta values for the blocking ELISA, for the same sera, were plotted for both the crude and purified antigens. All 3 plots showed a similar decrease in antibody reactivity to the viral antigen with time. The calculated *r* values, comparing the mean SN titers and the mean delta values for the blocking ELISA, were 0.11 and 0.67 for the crude and purified viral antigen, respectively.

A summary comparing SN titers and the results for the ELISA procedures on the same sera is given in Table 1. Viral antigen preparation (crude or purified) had some influence on the calculated *r* values. When purified viral antigen was used in the assay, sera from calves (Group A) vaccinated with BRSV had an *r* value of -0.39 compared to sera from unvaccinated calves with an *r* value of -0.14 (not shown in Table 1), but these values were still lower than the correlation values for the Group B sera. Both the indirect blocking ELISA and the indirect ELISA results gave better correlations with the SN test than those obtained with the blocking ELISA. The sensitivity was lowest (44%) for the indirect ELISA using the crude viral antigen and highest (81%) for the blocking ELISA using the purified viral antigen and sera from Group B calves. In general, specificity percentages were high and ranged from a low of

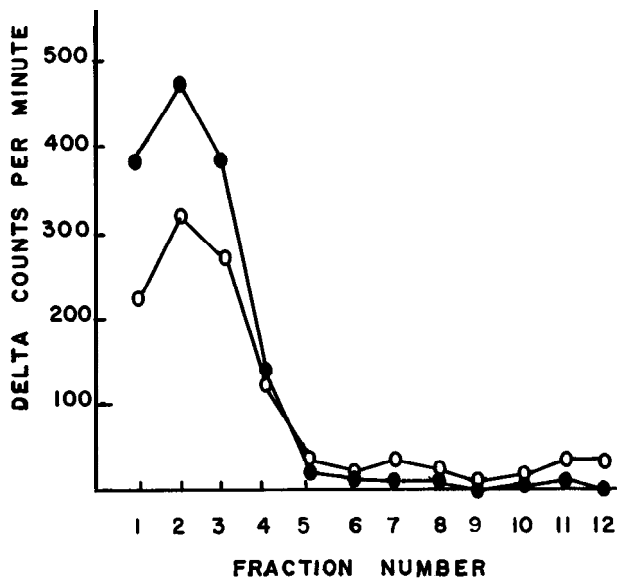


Figure 1. Indirect radioimmunoassays: sucrose density gradient fractions of Nonidet P-40-treated BRSV as antigens; hyperimmune gnotobiotic calf serum (78-16), or MAb 8G12 as the source of antibodies. Symbols: O, 78-16, 1:80; ●, MAb 8G12, 1:2,000.

75% for the indirect ELISA (purified viral antigen) to 100% for blocking ELISA (Group A sera, crude virus; Group B sera, crude or purified virus) and indirect ELISA (crude virus) as shown in Table 1.

### Discussion

One of the problems encountered early in the adaptation of the ELISA and the IRIA for detection of antibodies to BRSV was the nonspecific background of selected field sera. However, high background was not observed when hyperimmune gnotobiotic calf serum (78-16) was used in the indirect ELISA or IRIA. The results in Fig. 1 indicate that serum 78-16 reacted with the viral antigen in the density gradient fractions 1-12 in a manner similar to that by the MAb's to the fusion protein of BRSV (8G12) and basically only to antigens in fractions 1-3. However, bovine sera 0044 and 2806 contained antibodies which reacted with antigens in all of the fractions. These results indicate that the crude virus preparation contained antigens that reacted with antibodies, in some field bovine sera, that were not necessarily specific for BRSV. These antibodies may be directed toward carbohydrates (Christensen DL, Rhodes MB: 1988, Abstr Conf Res Workers Anim Dis #393). The purified virus contained fewer nonviral antigens but should not be considered a "pure" BRSV antigen since sera 0044 and 2806 for 2 bleeding dates, with large differences in SN titers, gave similar delta cpm in the IRIA (fractions 1-3, Fig. 2). However, since MAb 8G12 is directed only to the fusion protein of BRSV, the interference due to antibodies to nonviral antigens seems to be eliminated. This allows for the

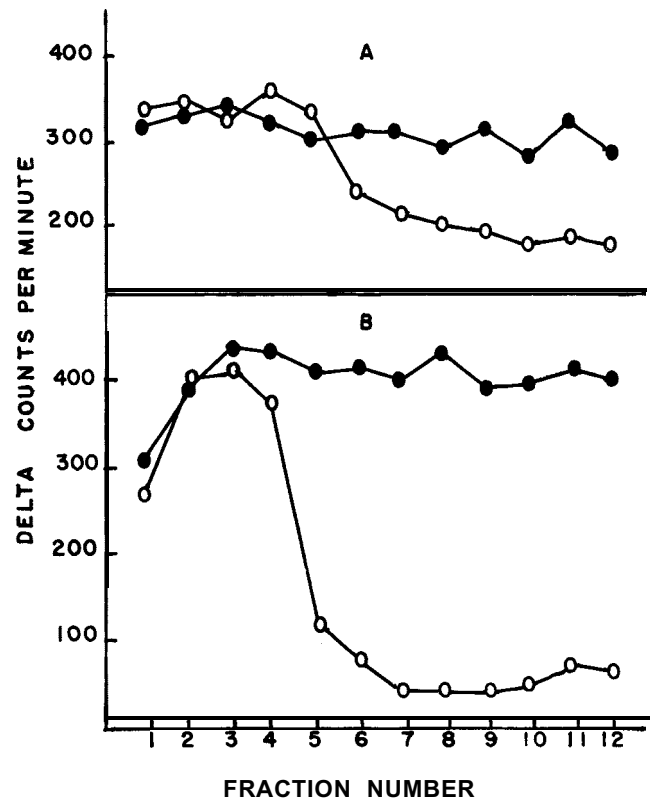


Figure 2. Indirect radioimmunoassay: antigen fractions as Fig. 1; field bovine sera 0044 and 2806 as the source of antibodies. Sera were diluted 1:80. Symbols: A, O, serum 0044, 11-18-85, SN titer = 1:640; ●, serum 0044, 12-7-85, SN titer = 1:20; B, O, serum 2806, 11-8-85, SN titer = 1:320; ●, serum 2806, 12-7-85, SN titer = 1:40.

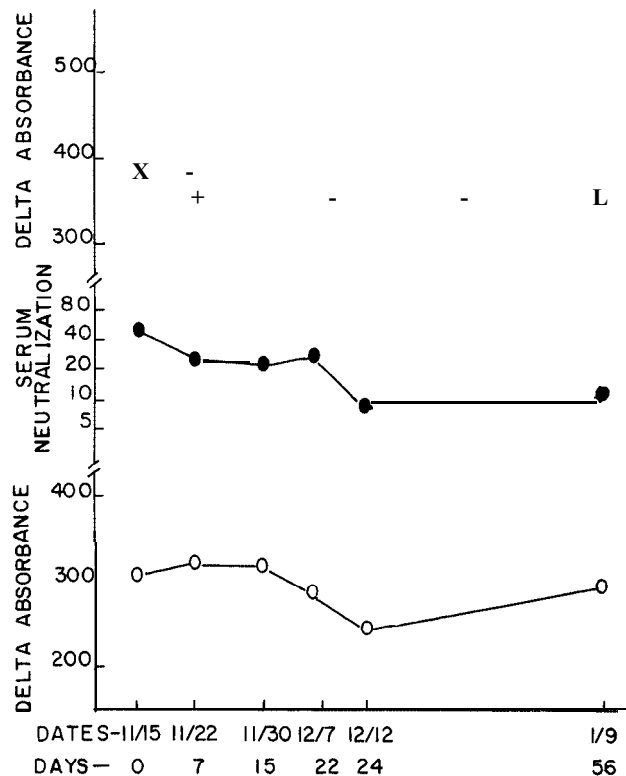
use of either crude or purified viral antigen in the blocking ELISA. The results in Table 1 indicate that this statement is true.

The blocking ELISA was relatively rapid and convenient to perform and should detect all classes of

Table 1. Comparison of the results of the blocking ELISA, indirect blocking ELISA, and indirect ELISA to the serum neutralization titers to BRSV for 159 field-origin bovine sera.

Sera group	No. of sera	Ag*	r	Sensitivity (%)	Specificity (%)
Blocking ELISA					
A	92	CV	-0.20	66	100
B	67	CV	-0.42	73	100
Weighted mean:			-0.31	70	100
A	92	PV	-0.22	52	83
B	67	PV	-0.56	81	100
Weighted mean:			-0.36	64	90
Indirect blocking ELISA					
A	28	PV	+0.65	61	75
Indirect ELISA					
A	92	CV	+0.48	44	100
B	92	PV	+0.51	68	92

\* Antigen: CV = crude viral antigen; PV = purified viral antigen; both used in the ELISA procedures. Correlation coefficient.



**Figure 3.** Plots of the mean values for SN titers or delta absorbance values for the blocking ELISA for each bleeding date from November 11, 1985 to January 9, 1986. Symbols: X, blocking ELISA-crude virus; ●, SN titers; ○, blocking ELISA-purified antigen.

antibodies that are specific to BRSV. The lack of correlation between the SN titers and the absorbance values obtained from the blocking ELISA suggested the assays measured antibodies to 2 different antigens, possibly the neutralization antigen(s) and the fusion protein, respectively. Monoclonal antibody 8G12 had a neutralization titer of 1:250 and an ELISA titer of > 1:12,000. Thus, our results suggest that the fusion protein of BRSV may not be the primary neutralization antigen(s).

The indirect blocking ELISA primarily measures antibodies to BRSV antigens other than the fusion protein since the latter should be blocked by the unlabeled MAb. Thus, this assay may measure neutralization and/or precipitating antibodies and could account for it having a higher correlation with the SN titers as the latter compared to the blocking ELISA. Similar points should also be relevant to the indirect ELISA, except this assay should also measure antibodies to the fusion protein. The correlation value between the SN titers and the indirect ELISA was 0.51 compared to 0.78 reported in the IRIA, both using the same purified antigen; however, only 25 of the sera from Group A calves were used in the IRIA study.

Fluorescein-labeled MAb 8G12 (anti-fusion protein) is currently being used for the detection of BRSV in

cell culture and animal tissues by a number of laboratories (C. A. Klucas, personal communication). Consequently, the use of HRPO-anti-fusion protein in the blocking ELISA, described above, would be expected to be specific for the virus and indirectly measure specific antibodies to BRSV. The blocking ELISA described here does not give values that correlate well with the SN titers on an individual animal basis; however, calculated sensitivities and specificities were relatively high when compared to the positive and negative titers by the SN test. In addition, the mean SN titers and the delta values for the blocking ELISA declined in a similar manner over a 56-day period. These characteristics plus the relative speed and lack of non-specific background of the blocking ELISA make this assay useful as a serological test for BRSV antibodies.

### Acknowledgements

The authors thank the following technical personnel: C. New, Jr., T. Sharp, D. Christensen, personnel in the Virology Section of the Veterinary Diagnostic Center, Virology Section, and personnel at the Roman Hruska USDA APLA Meat Animal Research Center, Clay Center, NE. This paper was published as Journal Series No. 8776 with the approval of the Agricultural Research Division.

### Sources and manufacturers

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- Model L-5 ultracentrifuge, Spinco Division, Beckman Instrument Co., Palo Alto, CA.
- Bronson Sonic Power, model S 125, Heat Systems, Inc., Danbury, CA.
- Falcon 3075, Becton Dickinson Labware, Becton Dickinson, Oxnard, CA.
- Microplate Reader, Dynatech Laboratories Inc., Chantilly, VA.
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