Sensitivity and Specificity of an Enzyme-linked Immunosorbent Assay for the Detection of Infectious Bovine Rhinotracheitis Viral Antibody in Cattle

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ABSTRACT

An enzyme-linked immunosorbent assay was developed to detect bovine serum antibody to infectious bovine rhinotracheitis virus. The specificity of this assay in 304 bovine sera, collected from an infectious bovine rhinotracheitis virus-free herd, was 100%; in sera from 62 cattle inoculated with an intranasal vaccine, its diagnostic sensitivity was 27.4% at one month and 100% at six months, postvaccination. In 303 bovine sera with standard serum neutralizing antibody titers of $\geqslant$1:2 it showed 100% sensitivity; and in 463 random diagnostic samples, comparative tests indicated that enzyme-linked immunosorbent assay detected more seropositive animals (61.6%) than the standard serum neutralizing test (49.9%). The enzyme-linked immunosorbent assay method was considered to be technically superior as a routine diagnostic test for the detection of infectious bovine rhinotracheitis viral antibody in bovine sera.

Key words: Infectious bovine rhinotracheitis, enzyme-linked immunosorbent assay.

RÉSUMÉ

Cette expérience consistait à utiliser la technique immunoenzymatique ELISA pour rechercher les anticorps contre le virus de la rhino-trachéite infectieuse bovine, dans le sérum de bovins. Elle s'avéra efficace à 100%, pour la détection des anticorps précités, dans 304 échantillons de sérum qui provenaient des sujets d'un troupeau exempt du virus de la rhino-trachéite infectieuse bovine. Son efficacité n'atteignit que 27,4%, lors de l'épreuve du sérum de 62 bovins, un mois après leur vaccination intranasale; six mois après cette vaccination, son efficacité atteignit cependant 100%. Elle démontra aussi une efficacité totale, lors de l'épreuve de 303 échantillons de sérum bovin dont le titre d'anticorps égalait ou dépassait 1:2 avec l'épreuve standard de séroneutralisation. Lors de l'épreuve de 463 échantillons de sérum choisis au hasard parmi ceux qui avaient été soumis pour diagnostic, la technique immunoenzymatique ELISA décela 61,6% des sujets séropositifs, comparativement à 49,9% pour l'épreuve standard de séroneutralisation. De tels résultats amenèrent les auteurs à admettre la supériorité de la technique ELISA comme épreuve routinière de diagnostic pour la recherche des anticorps contre le virus de la rhino-trachéite infectieuse bovine, dans le sérum de bovins.

Mots clés: rhino-trachéite infectieuse bovine, technique immunoenzymatique ELISA.

INTRODUCTION

The serum neutralization (SN) test is routinely used by Agriculture Canada for serodiagnosis of infectious bovine rhinotracheitis (IBR) virus infection in cattle for export certification purposes. The SN test is specific in terms of yielding negative results on samples collected from known noninfected animals, however, the sensitivity of this test is considered to be suboptimal and consequently may give false negative reactions. In addition the SN test requires expensive tissue culture systems and is time consuming. Recently, several reports on enzyme-linked immunosorbent assay (ELISA) for the detection of IBR viral antibodies have been published (1-3). This communication describes the application of an ELISA for the detection of IBR viral antibodies in bovine sera and measures the performance of the test in terms of its diagnostic specificity and sensitivity. The feasibility of adopting an ELISA for routine serodiagnosis of IBR virus is also discussed.

MATERIALS AND METHODS

SERA

Three hundred and four sera which had been collected from the Animal Diseases Research Institute (ADRI) Lethbridge cattle herd were used to determine the diagnostic specificity of the ELISA. With the exception of the occasional introduction of an IBR seronegative bull this has been a closed herd for seven years and no clinical evidence of IBR virus infection, based on repeated SN tests, has been observed during this period. The last outside animal was introduced 18 months ago and all animals in the herd have been IBR seronegative for four consecutive biannual tests. For the diagnostic sensitivity assay, 62 ten month old calves from another institutional herd were inoculated with a modified live intranasal IBR vaccine (Connaught Laboratories, Willowdale, Ontario) and serum samples were collected at one and six months postvaccination. In order to assess relative sensitivity, 303 sera, which had standard SN antibody at serum dilutions of

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1:2 or greater, were selected. Finally to compare ELISA with the standard and modified SN tests, 121 sera from an IBR virus-infected herd and 342 sera from animals of unknown infection status which had been submitted for diagnostic purposes, were used. A positive reference serum was prepared by pooling sera collected from ten IBR virus-infected cattle with demonstrable SN antibody, and a negative reference serum was obtained by pooling sera from five cattle in the noninfected herd.

SERUM NEUTRALIZATION TESTS

Standard SN and modified SN tests (4,5) were performed in a microsystem. Twofold serial dilutions (1:2-1:256) of heat inactivated test serum (56° C, 30 min) 0.05 mL were mixed with equal volumes of IBR virus, Colorado strain, containing 100 $TCID_{50}$. The mixtures were incubated for 1 h at room temperature for the standard SN and for 24 h at 37° C in a CO_2 incubator for modified SN. In the modified SN a final concentration of 5% guinea pig complement was added to the serum dilution. At the end of incubation, 0.05 mL of secondary bovine fetal kidney cells were added to the test wells and the microplates were further incubated for three days at 37° C in a CO_2 incubator. The highest dilution of serum which gave complete neutralization of the virus was recorded as the SN titer.

ELISA ANTIGEN PREPARATION

In a preliminary study three different methods of antigen preparation reported previously (1-3) were assessed. A Freon 113 (DuPont, Calgary, Alberta) purified antigen similar to that described by Herring *et al* (2) proved satisfactory and gave consistent results. Briefly, tissue culture fluid collected from Colorado strain or 108 strain of IBR virus-infected secondary bovine kidney cells at maximum cytopathic activity was clarified by centrifugation at 1000 g for 10 min. The supernatant was decanted and concentrated by ultracentrifugation at 40,000 g for 1 h. The pellets were resuspended to 1/50 of their original volume in NTE buffer (0.15M NaCl, 0.01M tris, 1 mM EDTA, pH 7.2). The virus suspension was treated with equal volume of Freon 113 for 2 min and the aqueous phase collected after centrifugation at 2000 x g for 10 min and used as the source of antigen. The concentration of the antigen was determined by titration with 1:100 dilutions of the reference positive and negative sera. The antigen dilution, which gave absorbance at 492 nm (A_{492}) $\geqslant$ 1.200 with a reference positive serum, was used as the working dilution of the antigen.

ELISA PROCEDURE

Antigen Coating — Linbro EIA microtitration plates with 96 flat bottom wells (Flow Laboratories, Hamden, Connecticut; Cat. No. 76-381-04) were used for the test. The working dilution of the antigen was made in 0.05M carbonate buffer with 0.125M NaCl at pH 9.5. The wells of the microtitration plates, columns 2 to 11 inclusive, were sensitized by adding 0.2 mL of the diluted antigen per well. The wells in columns 1 and 12 received 0.2 mL of the carbonate buffer and were used as various controls.

After incubation at 4° C overnight in a covered container, the wells were decanted, dried in air, wrapped in a plastic bag and stored at -63° C until used.

Test Sera — ELISA tests were performed in triplicate plates as shown in Figure 1. To eliminate nonspecific hydrophobic binding to the plate surface, 0.35 mL diluent [Dulbecco PBS, containing 0.5% Tween 20 and 1% heat inactivated (56° C 30 min) horse serum] was added to each well and incubated 10-15 min at room temperature, followed by three washings with tap water in a Titertek Microplate Washer (Flow Laboratories, Hamden, Connecticut). Sera were diluted 1:100 in the same diluent. Appropriate wells received 0.2 mL of the diluted sera after which the plates were incubated for 2 h at 37° C and then washed five times as above.

Peroxidase Conjugate — Optimum concentration of peroxidase-conjugated IgG fraction of rabbit antibovine IgG (heavy and light chains) was diluted in the diluent (e.g. 1:16,000 dilution with Lot No. 16668 conjugate, Cappel Laboratories, Cochranville, Pennsylvania). The conjugate, (0.2 mL per well) was allowed to react for 1 h at 37° C, followed by washing three times in the microplate washer. An optimum concentration of the conjugate is defined as the highest dilution of the conjugate which gave the highest positive/negative (P/N) ratio and gave an A_{492} value of $\geqslant$ 1.200 with a reference positive serum.

Substrate — Freshly prepared o-phenylenediamine, 1 mg/mL in 0.1M citrate buffer pH 5.5 with 3 mM H_2O_2 was used as the substrate. After the substrate (0.2 mL per well) had been incubated for 45 min at room temperature, 50 μL *of* 4*N* H_2SO_4 were added to each well to stop the reaction.

INTERPRETATION

The A_{492} value of each plate was read with a Titertek Multiskan Pho-

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	1	2	3	4	AC	33	34	35	36	AC	CC
B	St	5	6	7	8	R-	37	38	39	40	R+	S-
C	St	9	10	11	12	R-	41	42	43	44	R-	S-
D	St	13	14	15	16	R+	45	46	47	48	R-	S-
E	St	17	18	19	20	R-	49	50	51	52	R+	S+
F	St	21	22	23	24	R-	53	54	55	56	R-	S+
G	St	25	26	27	28	R+	57	58	59	60	R-	S+
H	St	29	30	31	32	AC	61	62	63	64	AC	CC

Plate 2

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	49	50	51	52	AC	17	18	19	20	AC	CC
B	St	53	54	55	56	R-	21	22	23	24	R+	S-
C	St	57	58	59	60	R-	25	26	27	28	R-	S-
D	St	61	62	63	64	R+	29	30	31	32	R-	S-
E	St	33	34	35	36	R-	1	2	3	4	R+	S+
F	St	37	38	39	40	R-	5	6	7	8	R-	S+
G	St	41	42	43	44	R+	9	10	11	12	R-	S+
H	St	45	46	47	48	AC	13	14	15	16	AC	CC

Plate 3

	1	2	3	4	5	6	7	8	9	10	11	12
A	St	21	22	23	24	AC	53	54	55	56	AC	CC
B	St	25	26	27	28	R-	57	58	59	60	R+	S-
C	St	29	30	31	32	R-	61	62	63	64	R-	S-
D	St	17	18	19	20	R+	49	50	51	52	R-	S-
E	St	13	14	15	16	R-	45	46	47	48	R+	S+
F	St	1	2	3	4	R-	33	34	35	36	R-	S+
G	St	5	6	7	8	R+	37	38	39	40	R-	S+
H	St	9	10	11	12	AC	41	42	43	44	AC	CC

Fig. 1. Diagram of ELISA test pattern on EIA Microtiter plates.
Numbers: test samples
St: substrate control
AC: antigen control
CC: conjugate control
R+, R-: reference positive and negative serum
S+, S-: reference positive and negative serum control.

tometer (Flow Laboratories). A P/N ratio was calculated by dividing the A_{492} value of each test sample replicate with the average A_{492} of reference negative sera for that plate and from the triplicate test an average P/N ratio was determined. The results were interpreted as follows:

P/N ratio <1.50
negative
P/N ratio $\geqslant 1.50 \sim < 2.00$
suspicious
P/N ratio $\geqslant 2.00$
positive

The performance of the ELISA was considered in terms of the following parameters as defined by Vecchio (6).

$$\text{Specificity (\%)} = \frac{\text{No. of ELISA negative samples}}{\text{all negative samples}} \times 100$$

$$\text{Sensitivity (\%)} = \frac{\text{No. of ELISA positive samples}}{\text{all positive samples}} \times 100$$

Repeat of Test — Infrequent unusual A_{492} values were not included in the calculation unless more than one of the triplicate tests was affected, in which case the sample was retested. The entire test was repeated if the A_{492} values of the reference sera deviated from expected values.

STATISTICAL ANALYSIS

Statistical tests included analysis of variance of the P/N ratios by randomized block design, and correlation coefficients between tests, by Pearson product-moment method (7).

RESULTS

ELISA ANTIGEN

Titration of three lots of Freon 113-purified IBR viral ELISA antigen is shown in Figure 2. With positive reference serum, higher concentrations of antigen gave progressively higher A_{492} values and higher P/N ratios over an antigen dilution range of 1:40 to 1:5120. At antigen dilutions of 1:10 and 1:20, plateau reactions were observed. With negative reference serum, the antigen dilutions did not affect either the A_{492} values or the P/N ratios. Both the Colorado strain and 108 strain of IBR virus, which both belong to restriction-endonuclease type 1A (8), produced satisfactory ELISA antigen. A group of negative (n = 162) and positive (n = 30) sera were tested with antigen dilutions of 1:50 and 1:300 (Table I). The correlation coefficients between the antigen dilutions for the negative and positive sera were 0.506 and 0.927, respectively ($P < 0.001$). Analysis of variance indicated that P/N ratios were significantly different between the dilutions at the 5% level with the negative sera and at the 0.1% level with the positive sera.

TABLE I. Effect of Antigen Concentration on Positive/Negative Ratio

Sera	Antigen dilution	Positive/Negative ratio[a] Mean ± S.E.	Minimum	Maximum	Correlation[b] coefficient
Negative	1:300	1.024 ± 0.009	0.610	1.460	
(N = 162)	1:50	1.002 ± 0.009	0.760	1.440	0.506
Positive	1:300	4.649 ± 0.240	2.870	7.440	
(N = 30)	1:50	5.785 ± 0.432	3.140	11.710	0.927

[a]Analysis of variance of P/N ratios are significant at 5% level with negative sera and 0.1% level with positive sera
[b]All correlation coefficients are significant at 0.1% level

TABLE II. Specificity Test of the ELISA Using Bovine Sera Collected from an IBR Virus Free Herd

Date of bleeding	No. of samples	ELISA test P	S	N	Average P/N ratio ± S.E.	Specificity (%)
February 3, 1983	23	0	0	23	1.13 ± 0.040	100
June 13, 1983	77	0	0	77	1.04 ± 0.022	100
August 30, 1983	32	0	0	32	1.07 ± 0.028	100
October 18, 1983	10	0	0	10	1.06 ± 0.060	100
November 22, 1983	162	0	0	162	1.02 ± 0.013	100
Total	304	0	0	304	1.04 ± 0.010	100

P = positive
S = suspicious
N = negative

SPECIFICITY AND SENSITIVITY OF ELISA

All 304 sera collected from the IBR virus-free herd showed P/N ratios of less than 1.5 and revealed 100% specificity of the test (Table II). All of these sera were also negative with both the standard and modified SN tests. The sera of the 62 calves which were given an intranasal vaccine showed an ELISA test sensitivity of 27.4% and 100% at one month and six months postvaccination, respectively (Table III). All 303 samples which had a titer of $\geqslant$ 1:2 in the standard serum neutralization test gave P/N ratios in excess of 2.0 and a sensitivity of 100% (Table IV).

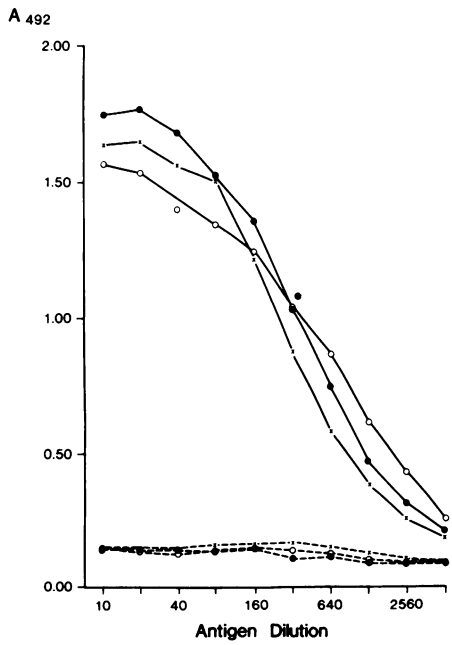

Fig. 2. IBRV-ELISA antigen titrations with negative (broken line) and positive (solid line) reference sera; o--o •--• Colorado strain of IBRV; x--x 108 strain of IBRV.

TABLE III. Comparison of ELISA with Standard SN Test on IBR Virus Intranasally Vaccinated Cattle

Sample	ELISA test				SN test		
	P[a]	S	N	% Sensitivity	P	N	% Sensitivity
Before vaccination (ten month old)[b]	1	0	61		0	62	
One month postvaccination	17	6	39	27.4	13	49	21.0
Six month postvaccination	62	0	0	100	59	3	95.2

[a]P = positive, S = suspicious, N = negative
[b]Majority of these animals, 35 of 37 tested, had maternal antibody against IBR virus at six weeks of age

TABLE IV. Relative Sensitivity of the ELISA Using Standard SN-Positive (≥1:2) Cattle Serum

Sample origin	ELISA			Sensitivity (%)
	P	S	N	
Export test	58	0	0	100
Quality control	74	0	0	100
Intranasal vaccinates	72	0	0	100
Infected herd	99	0	0	100
Total	303	0	0	100

P = positive
S = suspicious
N = negative

TABLE V. Test of Repeatability of Positive/Negative Ratio

Variable	Positive/Negative ratio[a]			Correlation coefficient[b]			
	Mean ± S.E.	Minimum	Maximum	Day 1	Day 2	Day 6	Day 9
Negative sera (N = 31)							
Day 1	1.034 ± 0.025	0.820	1.385				
Day 2	1.001 ± 0.028	0.710	1.350	0.8166			
Day 6	1.030 ± 0.024	0.785	1.350	0.8010	0.7060		
Day 9	0.970 ± 0.023	0.730	1.305	0.6602	0.6425	0.8103	
Day 12	0.938 ± 0.024	0.690	1.370	0.7948	0.7575	0.8885	0.8230
Positive sera (N = 38)							
Day 1	5.266 ± 0.453	2.115	11.710				
Day 2	5.312 ± 0.455	2.455	12.100	0.9916			
Day 6	4.623 ± 0.427	1.705	11.830	0.9726	0.9803		
Day 9	5.311 ± 0.862	1.810	12.665	0.9897	0.9865	0.9669	
Day 12	5.136 ± 0.468	1.830	12.190	0.9930	0.9915	0.9681	0.9925

[a]Analysis of variance of P/N ratios of repeated tests of both negative and positive sera are significant at the 1% level
[b]All correlation coefficients are significant at the 0.1% level

TABLE VI. Comparison of ELISA with Standard and Modified Serum Neutralization Tests

Source of Sample	ELISA			Standard SN		Modified SN		Total
	P	S	N	P	N	P	N	
Quality control	76	2	42	74	46	78	42	120
Export testing	109	22	91	58	164	109	113	222
Infected herd	100	2	19	99	22	104	17	121
Total	285	26	152	231	232	291	172	463

ELISA reaction	No.	Standard SN		Modified SN	
		P	N	P	N
P	285	231	54	274	11
S	26	0	26	14	12
N	152	0	152	3	149
Total	463	231	232	291	172

P = Positive
S = Suspicious
N = Negative

REPEATABILITY OF ELISA

Aliquots of 38 positive sera with various P/N ratios, including nine sera with borderline reactions, and 31 negative sera were tested at five different dates (Table V). All the negative sera showed negative ELISA results throughout the five repeat tests. All the positive sera showed positive reactions throughout the five repeat tests with the exception of one on day 6 and two on days 9 and 12 which showed suspicious reactions. These three sera had been selected from lower titer samples. Correlation coefficients between all combinations of repeated tests are also given in Table V. All correlation coefficients in Table V are significant at the 0.1% level. Positive sera gave superior correlations between the tests.

COMPARISON OF ELISA WITH STANDARD AND MODIFIED SN TESTS

Four hundred and sixty-three sera were used for comparison of the ELISA with the two SN tests (Table VI). The ELISA results showed 285 to be positive, 26 suspicious and 152 negative. The standard SN test detected 231 sera as positive and 232 sera as negative whereas the modified SN test showed 291 sera positive and 172 sera negative. All 231 sera which were standard SN positive were also positive both with the ELISA and the modified SN test. Of the 285 ELISA positive sera 274 were modified SN positive and 11 were negative, whereas of 291 sera which were positive with the latter test 274 were ELISA positive, 14 suspicious and three negative (Table VI). Figure 3 illustrates the P/N ratio frequency distribution of 304 negative sera which were assayed in Table I and 282 of 291 modified SN positive sera in Table VI (nine positive sera were duplicate samples in quality control test). Based on end point titrations, the modified SN test, on average was about 15 times more sensitive than the standard SN in detecting IBR viral neutralizing antibody.

DISCUSSION

The data presented indicate that the

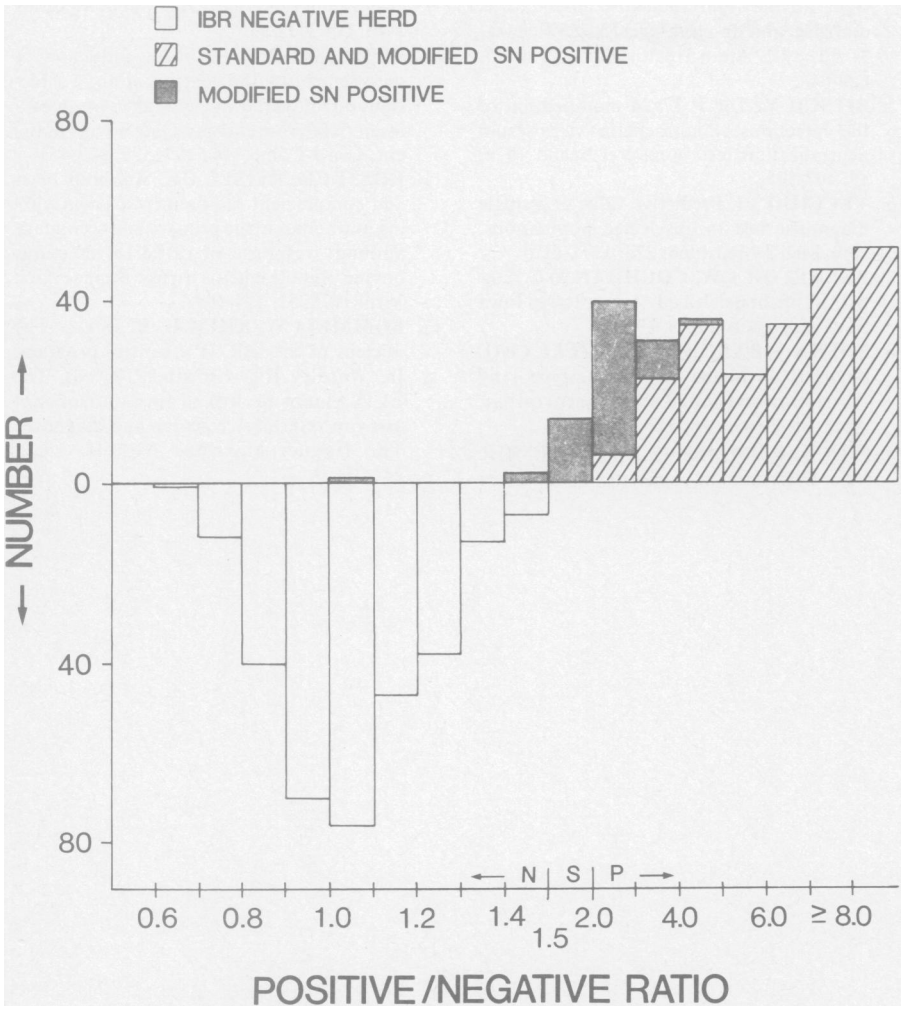

Fig. 3. Frequency distribution of P/N ratios of ELISA with sera collected from IBRV negative herd (n = 304) and modified SN test-positive sera (n = 282).

ELISA is a specific, sensitive and practical test for the detection of anti-IBR viral antibodies. This conclusion is in agreement with previous reports (1-3).

Many workers have determined the cut-off value of absorbance from known negative sera. As negative sera are usually selected by assays less sensitive than ELISA, sera with undetected antibody titers may be included in the reference negative serum pool thus increasing the possibility of false negative results (9). To avoid this limitation in the present study, the specificity of ELISA was determined by using a large number of sera (n = 304) collected from a known IBR virus-free herd. This should provide a more accurate indication of the diagnostic specificity of the test. Five of these sera were also used as reference negative pool in subsequent tests. Also, to reduce the inherent inter- and intra-group variations of A_{492} readings among plates and test dates, three replicate samples were tested on three separate plates at three different sample placement locations (Figure 1) and the P/N ratio was used as the cut-off value. All 304 sera collected from the IBR virus-free herd showed P/N ratios of less than 1.5 and all 303 samples which had a titer of $\geqslant$1:2 in the standard serum neutralization test gave P/N ratio of in excess of 2.0. Based on these data, a criterion for the interpretation of positive (P/N ratio $\geqslant$2.00) and negative (P/N ratio $<$1.50) was established. For those sera which were neither positive nor negative (P/N ratio $\geqslant$1.50 - $<$2.00), suspicious interpretations were given. These suspicious reactions are borderline and may represent very low concentrations of serum antibody produced in the early stage of IBR virus infection, which would subsequently become ELISA positive as shown in Table III. We have also observed suspicious reactions in sera used in a maternal antibody decay study, which subsequently become ELISA negative (Cho, Bradley and Yates, unpublished observations). In the present analysis of diagnostic sensitivity and specificity the suspicious results were considered as neither positive nor negative.

Based on the results obtained, the ELISA method appears to be superior to the standard SN test which is the official serological test for the detection of IBR viral antibody for regulatory purposes in Canada. Both tests have been shown to be specific, however, the ELISA method is more sensitive and practical. The present ELISA shows similar sensitivity to the modified SN test. In comparing the standard and modified SN tests, the latter proved to be on the average of about 15 times more sensitive than the former in detecting antibody titers and it allows the detection of complement-dependent IBR viral antibodies (5,10,11).

Due to its many advantages over the SN test, such as its sensitivity, its convenient technical features (1-3) plus its potential adaptability for automation, the ELISA method may be efficiently utilized as an alternative official test for the detection of IBR viral antibodies in cattle. In Switzerland, a micro-ELISA has been extensively utilized to detect IBR antibody (12).

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