A Comparative Evaluation of Two Sensitive Serum Neutralization Tests for Bovine Herpesvirus-1 Antibodies

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ABSTRACT

Two sensitive serum neutralization (SN) tests for the detection of antibodies to bovine herpesvirus-1 (BHV-1) in bovine sera were evaluated. Both SN tests used a 24 h incubation of test sera with 100 $CCID_{50}$ of BHV-1 before the addition of susceptible cells. The tests differed in the presence (C test) or absence (D test) of complement and were compared with a standard 1 h incubation SN test and the enzyme-linked immunosorbent assay (ELISA). Although the mean titer of the C test was twofold higher than the mean titer of the D test for 310 sera, the number of samples which were negative was not significantly different between tests. For 100 sera from herds with known reactors, which were negative in a 1 h incubation SN test, 32% tested positive in the C and D tests. Other investigations, including Western immunoblotting and radioimmune precipitation, suggest that the 24 h incubation tests produce some false positive results. In contrast, the 1 h incubation SN test and, to a much lesser extent, the ELISA appear to produce some false negative results. The C test was more sensitive than the D test for detecting an early immune response after experimental infection.

RÉSUMÉ

Deux tests de séroneutralisation (SN) pour la détection d'anticorps sériques contre le virus herpès bovin-1 (VHB-1) ont été évalués. Les deux tests comportaient une pré-incubation de 24 h avec 100 $CCID_{50}$ de VHB-1 avant l'ajout de cellules sensibles. Les tests différaient selon la présence (test C) ou l'absence (test D) du complément et ont été comparés avec un test étalon comportant un test de SN d'une heure et un test ELISA. Quoique le titre moyen du test C ait été deux fois supérieur à celui du test D pour 310 échantillons, le nombre d'échantillons négatifs n'était pas significativement différent. Pour 100 échantillons provenant de troupeaux infectés, lesquels étaient négatifs au test de SN d'une heure, 32 % furent positifs aux tests C et D. D'autres analyses incluant l'immunobuvardage et le dosage radioimmunologique, ont démontré que 24 h d'incubation produisait quelques faux positifs tandis que le test de SN d'une heure, et à un degré moindre le test ELISA, produisaient quelques faux négatifs. Le test C s'est montré plus sensible que le test D afin de détecter une réponse immunitaire suite à une infection expérimentale. (*Traduit par Dr Pascal Dubreuil*)

In the diagnosis of bovine herpesvirus-1 (BHV-1, infectious bovine rhinotracheitis virus) infections, the serum neutralization (SN) test and the enzyme-linked immunosorbent assay (ELISA) have been commonly employed to detect serum antibodies to the virus. For the exportation of animals and semen, cattle may be required to be antibody negative, i.e. never infected and thus not latently infected; or alternatively if vaccinated, show no rise in antibody titer, i.e. not acutely infected or showing reactivation of latent virus. In addition, the type of test (SN or ELISA) required is often specified by the importing country.

It is possible that some animals latently infected with BHV-1 will have very low antibody titers to the virus, and may even become seronegative, if they are not stressed and virus reactivation does not occur over a long period of time (L.A. Babiuk, personal communication). To identify animals with low titers it is essential to have tests that are sufficiently sensitive.

Two principal factors have been shown to influence the sensitivity of the SN test for the detection of BHV-1 antibodies in bovine serum; the length of incubation time between the virus and serum before the addition of cells, and the use of complement in the assay (1–4). Previously, a 24 h incubation SN test was found to yield higher titers than a 1 h incubation SN test (1). Further, Potgieter (2) found that bovine IgM antibody was dependent on complement for neutralization of BHV-1 while the same dependency was not observed with IgG antibody. In contrast, Rossi and Kiessel (3) found that both IgM and IgG produced during the first month after infection were primarily complement-requiring neutralizing antibody, but especially IgM. Further, Cho and Bohac (4) found that a 24 h incubation SN test with complement was more sensitive than a 1 h incubation test without complement. In this study, we made a comparative evaluation of two 24 h incubation SN tests, with (C test) or without complement (D test), to determine which of these SN tests could best detect low BHV-1 antibody titers.

Twofold serial dilutions of heat-inactivated serum (56°C, 30 min) were incubated with equal volumes

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(0.025 mL) of the Colorado strain of BHV-1 containing 100 $CCID_{50}$ for the 1 h standard (S) and 24 h incubation D SN test. [The latter test is similar to one used currently by the National Veterinary Laboratory in Denmark (V. Palfi, personal communication) and is thus referred to as the D (Danish) test]. Alternatively, 0.025 mL of reconstituted guinea pig complement (ADRI, Nepean, Ontario), was added to the serum dilutions at 5.0% final concentration, before virus was added for the 24 h incubation C SN test. The mixtures were then incubated for 1 h (standard test) or 24 h (C and D tests) at 37°C in a CO_2 (5%) incubator. At the end of incubation, 0.050 mL of trypsinized Madin Darby bovine kidney cells in Eagle's minimum essential medium (Gibco Canada Inc., Burlington, Ontario) containing 20 μg/mL gentamycin and 5% horse serum (Gibco, 2.5% final concentration) were added to the test wells. All samples (in duplicate) were further incubated for three days at 37°C in a CO_2 incubator. The highest dilution of serum which gave complete neutralization of virus, as determined by the absence of cytopathic effect, was recorded as the SN titer. The ELISA was performed as previously described using the 108 strain of BHV-1 (4). Relative sensitivity of a SN test refers to the percentage of ELISA positive animals that were tested positive in the SN test. Specificity refers to the percentage of noninfected cattle that gave a negative test.

Sera from 310 export beef and dairy cattle were tested (Table I). Both the C and D tests had a relative sensitivity of 100%. The mean titer for these sera by the C test was twofold higher than the mean titer of the D test ($p<0.001$) based on an analysis of variance (5) of the $\log_2$ reciprocal titer data. However, there was no significant difference between these tests ($p>0.05$) in the percentage of sera which tested negative (8.1% and 8.7%, respectively) based on McNemar's test (6). Thirty-three sera, or 10.7%, were negative by ELISA. This percentage was significantly higher ($p<0.05$) than the C test but not the D test. The relative sensitivity of the standard SN test was 98.5%. The standard SN test yielded significantly more (12.9%) negative results than the C and D tests ($p<0.01$) and the ELISA ($p<0.05$). The mean titer of the standard SN test was about eightfold less than the C test.

TABLE I. Distribution and mean neutralization titers of 310 bovine sera[a]

SN test:	S	D	C	
Complement:	−	−	+	ELISA
Negative (<1/2)	40	27	25	33
1/2	57	10	6	
1/4	95	13	15	
1/8–1/16	69	131	73	
1/32–1/64	49	102	100	
1/128–1/256	0	25	85	
⩾1/512	0	2	6	
Mean[b]	2.8	4.6	5.6	

[a]Sera was incubated with virus for 1 h (S) or 24 h (D,C) in the presence (+) or absence (−) of complement

[b]Mean of positive values (positive in all three SN tests) following $\log_2$ transformation of reciprocal titers. Standard error of the mean = 0.04 (538 df). The effects of sera and SN test were included in the statistical model. All means were significantly different from each other ($p<0.001$)

In addition to the tests described in Table I, the same sera were used to examine the effect of the cell culture system on the standard SN test, using secondary bovine kidney cells and calf serum (10%, final concentration) as the medium supplement (S_a test). Although the small difference in the mean ($\log_2$ reciprocal) titer (2.9 ± 0.02 vs 2.8 ± 0.02) was significant ($p<0.01$), there was no statistical difference in the percentage of sera (13.2%) which tested negative when this cell culture system was used for the standard test.

One hundred and four serum samples from the BHV-1-free herd at the Animal Diseases Research Institute (Lethbridge) were also tested. This herd has been monitored for BHV-1 infection by ELISA each trimester and has maintained its BHV-1-free status for several years (7). All sera were negative by the S SN test and ELISA (specificity = 100%), however, one serum was positive (titer = 1/2) by both the C and D tests (specificity = 99.0%). This animal was given repeated dexamethasone injections, as described (8), in an attempt to reactivate virus from a possible latent BHV-1 infection. However, virus could not be isolated, nor was there serological evidence of virus reactivation. This result indicated that the 24 h incubation SN tests may give some false positive results.

To determine which 24 h incubation SN test could best detect low levels of BHV-1 antibodies, 100 bovine sera, which were previously determined to be negative by a 1 h incubation SN test (S_a test), were examined. These sera originated from animals from seropositive herds. The results showed no difference in the percentage of sera (32%) which were positive in C and D tests (Table II). Significantly fewer ($p<0.01$) samples (12%) were ELISA positive or suspicious.

To determine whether the 24 h incubation SN tests were detecting BHV-1 specific antibodies when ELISA gave negative results or whether another factor may have been causing neutralization of virus in the assay, a proportion of these 100 sera were examined for the presence of BHV-1 antibodies by Western immunoblots (WB) and radioimmune precipitation assays (RIPA). All sera (n=34) positive in either C or D test, reference positive and negative (from the BHV-1-free herd) sera and several randomly selected sera (16 in total) which were negative by both C and D SN tests and ELISA, were examined. For WB, BHV-1 (Colorado strain) was purified and proteins were extracted essentially as described using 5% NP-40 (9,10). After separation of solubilized proteins by electrophoresis in polyacrylamide gels they were transferred to nitrocellulose membranes in a BioRad (Mississauga, Ontario) semidry transblot cell at 12V for 24 min as per manufacturer's instructions. Immunoreactions with diluted (1/2) sera (60–150 μL) were performed overnight at 4°C in a Miniblotter 16 or 28 (Immunetics, Cambridge, Massachusetts) after

TABLE II. Distribution of sera tested positive from 100 standard 1 h SN test negative animals from seropositive herds

SN titer[a]	D test	C test	ELISA	WB and RIPA[b]
1/2	10	13	1,1[c]	2,1[c]
1/4	11	5	5,3	5,4
1/8	8	7	3,3	5,4
1/16	3	7	3,5	3,6
Totals	32	32	12	15

[a]For D and C tests. A total of 34 sera gave a titer in either the D or C test (each SN test determined two sera positive which were negative in the alternate SN test)
[b]All sera were not tested by WB and RIPA; see text
[c]No. of sera positive (positive or suspicious for ELISA) of those that were positive in the D test, followed by the no. of sera positive (positive or suspicious for ELISA) of those that were positive in the C test

TABLE III. Animals testing positive for antibodies to BHV-1 after experimental infection (n=10)[a]

Test:	D	C	ELISA
Complement:	−	+	
Day 5[b]	0	0	0
Day 6	0	1	0
Day 7	0	8[c]	0
Day 10	10	10	10

[a]Seronegative animals from a BHV-1 free herd were infected with BHV-1 on day 0. Since animals were infected, a titer of ⩾ 1/2 was considered positive for both C and D tests
[b]Days after experimental infection; sera were not collected on days 8 and 9
[c]Results for the C test were significantly different ($p<0.05$) on day 7 from the other two tests

blocking with a 3–5% horse serum (Gibco) in PBS and 0.5% Tween 20 (PBST). After washing with PBST, blots were incubated with a 1/1000 dilution of biotinylated rabbit anti-bovine IgG (Sigma Chemical Co., St. Louis, Missouri) and again washed. After incubation with streptavidin conjugated with horseradish peroxidase (Amersham, Oakville, Ontario), reactions were detected with 4-chloro-1-naphthol (BioRad) as per manufacturer's instructions. The RIPA was performed with ^{35}S-methionine labelled BHV-1-infected cell lysates with 20 μL of sera essentially as described except that the RIPA buffer contained in addition to other detergents 0.5% SDS and 0.1 mM EDTA (11).

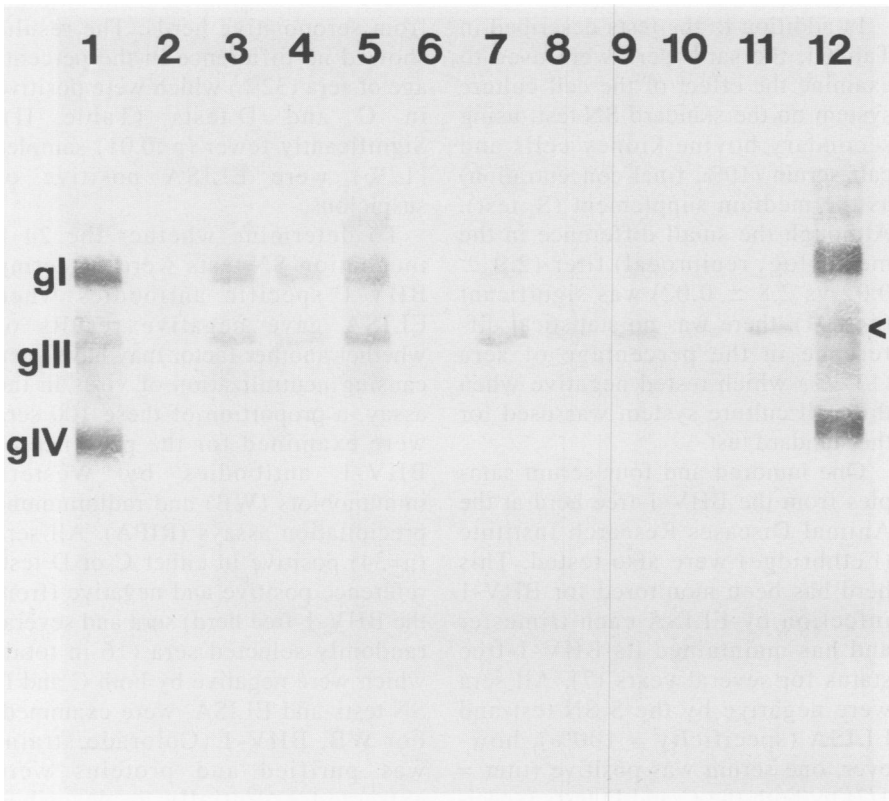

Fig. 1. Demonstration of BHV-1 specific antibodies in some low titer SN sera by Western immunoblotting. Proteins of BHV-1 (gI, gIII, gIV) were transferred onto membranes and reacted with sera before detection using biotinylated antibovine Ig, streptavidin-horseradish peroxidase and substrate. Lanes 1 and 12, reference positive antiserum. Lane 2, reference negative serum from BHV-1-free herd. Strong reactions with BHV-1 gI protein were observed with SN and ELISA positive sera #12, #36, #113 (lanes 3–5, respectively). Light reaction to gI was observed with SN positive, ELISA negative sera #165, #108, #112 (lanes 7–9, respectively). Negative reactions to gI were observed with SN positive, ELISA negative serum #114 (lane 10) and SN negative, ELISA negative sera #135 and #29 (lanes 6 and 11, respectively). An unidentified protein, which may be a contaminant, was observed within the BHV-1 gIII band (denoted by the arrow on the right). It was observed to react in several assays (including RIPA) with field negative sera (as in lane 11) and with the reference negative serum (not shown). Reactions to this protein were not scored. All test sera were negative by a standard 1 h SN test.

In WB, several blocking agents, including horse serum, bovine serum albumin and skim milk powder were examined. Horse serum (3–5%) performed best, although it was necessary to allow some background reaction to occur in order to retain sufficient sensitivity in the assay. Nevertheless, for a proportion of sera with low SN titers by the C and D tests (Table II), we observed reactions above those with negative sera and these were scored as positive reactions (Fig. 1). For 32 sera which gave low titers by C and D tests when a standard 1 h SN test gave negative results, 15 appeared to be positive for BHV-1 antibodies by WB and in an initial or repeated RIPA (Table II). Specific reactions were primarily directed to the BHV-1 gI protein which was shown previously to induce the earliest and most consistent immune response (12). Of the twelve sera which were ELISA positive or suspicious (Table II), all six ELISA positive sera and three of six sera which were suspicious also appeared positive for BHV-1 antibodies in both WB and RIPA. In addition, six sera that were ELISA negative appeared to be positive in both assays.

It was not possible from this study to calculate with accuracy, proportions of false positive and false negative

reactions for the SN tests and ELISA for several reasons. First, in WB and RIPA it would have been difficult to distinguish weak reactions from background reactions. It was also possible that some SN and ELISA negative sera contained low levels of nonneutralizing antibody that reacted in WB and RIPA but were attributed to background reactions. Further, all SN and ELISA negative sera were not examined, and perhaps, most importantly, the 100 sera were selected on the basis that they were negative by a 1 h SN test and came from herds which had reactors and thus excluded animals from seronegative herds. This selection was purposely done to increase the probability of obtaining weak reactors in the 24 h SN tests for test comparisons on sensitivity.

Although titers as high as 1/8–1/16 were obtained in C and D tests for standard SN test negative sera (Table II), a majority of sera with these titers will be standard SN test positive. For example, from 73 sera from the 310 export animals (Table I) which had titers of 1/8–1/16 in the C test, 70 (96%) were positive by the 1 h S test. For sera with titer of a 1/4 in the C test (in Table I) 60% (9/15) were positive in the 1 h SN test.

Most 1 h SN test negative sera with titers of 1/4–1/16 in the C test were positive for BHV-1 antibodies by WB and RIPA (Table II). A higher porportion of sera with these titers would probably have been found positive in WB and RIPA if 1 h SN test positive sera had been included. Of interest, all six sera in Table I with a titer of 1/2 in the C test were negative in the S SN test and the results from Table II suggest that most of these sera would be negative for BHV-1 antibodies as determined by WB and RIPA. Thus although the data is limited, it might be concluded that a titer of $\geq$1/4 in the C test for any serum is probably a true positive, whereas a titer of 1/2 is likely a false positive.

Similarly, for the D test 98% (129/131) of those sera (from Table I) with titers of 1/8–1/16 and about 70% (9/13) of the sera with a titer of 1/4 in the D test were S SN test positive. However, in contrast with the C test, three from ten sera with a titer of 1/2 in the D test (in Table I) were also positive in the S SN test. A similar analysis with data from Table II suggests that a significant proportion of sera with a titer of 1/2 in the D test may contain BHV-1 antibodies.

Finally, to determine which 24 h incubation SN test could best detect BHV-1 antibodies early after an infection, sera from ten animals which had been inoculated experimentally with BHV-1 were tested. Since seronegative, BHV-1-free animals were infected, a titer of $\geq$ 1/2 was considered positive for both C and D tests. While neither the D test nor the ELISA detected antibodies to BHV-1 on days 6 and 7 after experimental infection, the C test detected BHV-1 antibodies in one animal and eight animals, respectively, on these days (Table III). Titers of $\geq$ 1/16 and $\geq$1/512 were obtained on day 10, respectively, for the D and C tests. Thus, the C test was more sensitive in detecting BHV-1 antibodies early after an infection. This was expected, as an early response would constitute an IgM antibody response and neutralization of virus by IgM antibodies has been shown to be complement dependent (2,3). From the titers shown on day 10 which were as high as 1/256 for the D test, we presume that some animals would have been determined to be antibody positive by this test as early as day 8 if this sera had been collected and tested. Thus, compared to the D test and ELISA the C test may give two days of advanced confirmation of BHV-1 infection in previously negative animals.

In summary, the C test yielded higher titers and did detect BHV-1 specific antibodies earlier after infection than the D test, but both tests gave similar numbers of positive and negative results and both appear to produce some false positive results.

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