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A Simple, Specific, and Highly Sensitive Blocking Enzyme-Linked Immunosorbent Assay for Detection of Antibodies to Bovine Herpesvirus 1

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By using a monoclonal antibody directed against an epitope located on glycoprotein B of bovine herpesvirus 1 (BHV1), a simple, convenient blocking enzyme-linked immunosorbent assay (ELISA) which combines a high sensitivity with a low false-positive rate has been developed. The test can be performed at low variance on undiluted bovine serum samples. The epitope on glycoprotein B appears to be conserved, because it could be detected by immunostaining in all of 160 BHV1 isolates originating from 10 countries. In testing 215 anti-BHV1 antibody-negative and 179 anti-BHV1 antibody-positive serum samples, specificity and sensitivity were 0.96 and 0.99, respectively. This blocking ELISA is superior to a commercially available indirect ELISA and to the 24-h virus neutralization test in detecting low antibody levels in serum. In addition, this blocking ELISA is able to detect specific antibodies in serum as early as 7 days postinfection. To minimize any risk of introducing latent BHV1 carriers among noninfected cattle, this blocking ELISA would be, in our opinion, the test of choice.

Bovine herpesvirus 1 (BHV1), a member of the subfamily *Alphaherpesvirinae*, can cause a wide variety of syndromes, including infectious bovine rhinotracheitis, infectious pustular vulvovaginitis, infectious pustular balanoposthitis, abortion, conjunctivitis, and encephalitis (26). Moreover, BHV1 infections may be complicated by secondary bacterial infections (33). Following infection, latency of the virus is established (22). As a result of stressful conditions, the latent virus may be reactivated and shed at irregular intervals. Animals once infected must therefore be regarded as lifelong potential shedders of BHV1. Vaccination of animals does not uniformly prevent the establishment of latency (31). Herd-to-herd transmission of BHV1 most commonly occurs by introduction of infected animals into noninfected herds (22).

To diagnose infection of BHV1, serologic tests for specific antibody detection are used. BHV1 infections via the respiratory route normally induce a considerable and long-lasting titer of antibodies in serum after 7 to 10 days. Some reports suggest that there are (latently) infected cattle with no or only a minimal antibody response against the virus (6, 8, 12, 21). However, in most of these studies rather insensitive antibody detection methods have been used.

Because detection of latent virus carriers is of importance in control programs and international trade activities, tests to detect specific antibodies in serum must be highly sensitive (1). In the study presented, a simple, specific, and highly sensitive blocking enzyme-linked immunosorbent assay (ELISA) using BHV1 antigen-coated microtiter plates, undiluted bovine serum, and a monoclonal antibody (MAb) peroxidase conjugate directed against the glycoprotein B (gB) of BHV1 is described.

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MATERIALS AND METHODS

MAbs. BALB/c mice were immunized intraperitoneally with 100 μg of BHV1 (strain Lam: Dutch strain isolated in 1972) mixed with Freund's complete adjuvant. Immunization was repeated 30 days later. After another 30 days, mice were boosted intravenously with 50 μg of virus for 3 consecutive days, after which spleen cells were obtained. Production of MAbs was performed as described previously (29). Determination of subclass and light-chain type of MAbs was performed with an isotyping kit (ScreenType, catalog no. 100 035; Boehringer Mannheim Biochemicals).

The specificities of anti-BHV1 gB MAb 42/18/7, anti-BHV1 gC MAb 118/2/4, and anti-BHV1 gD MAb 21/3/3, used as reference MAbs in the radioimmunoprecipitation analyses (RIPAs), were ascertained on basis of their reactivity with Madin-Darby bovine kidney (MDBK) cells infected with recombinant vaccinia viruses expressing BHV1 glycoproteins gB, gC, and gD. For the expression of BHV1 gB, a 3.6-kb *Not*I fragment from the BHV1 strain Schönböken was used. This *Not*I fragment starts 246 bp upstream of the gB open reading frame (20) and was cloned into the *Eco*RI site of the vaccinia virus recombination vector VacGS43 (2). For the expression of BHV1 gC, a 3.2-kb *Bam*HI fragment from the same strain was used. This *Bam*HI fragment starts 62 bp upstream of the gC open reading frame (7) and was cloned in the *Sma*I site of VacGS43. Using the constructed vectors with the open reading frames downstream of the 7.5 promoter, the gB and gC open reading frames were recombined into vaccinia virus strain WR, according to published procedures (16, 17). The construction of the vaccinia virus-BHV1 gD construct has been described by Denis et al. (5).

IPMA. To detect BHV1-specific MAbs, an immunoperoxidase monolayer assay (IPMA) was performed essentially as described previously (29): confluent monolayers of embryonic bovine trachea (EBTr) cells in 96-well microtiter plates were inoculated with 100 μl of BHV1 (Lam strain; 10^4 50% tissue culture infective doses [$TCID_{50}$]/ml) dissolved in medium. When cytopathic effect (CPE) appeared, the medium was

discarded and plates were washed with phosphate-buffered saline (PBS), dried at 37°C for 1 h, and frozen at −20°C. Then, cells were fixed for 10 min with 100 μl of 4% (wt/vol) cold paraformaldehyde. Plates were washed with PBS and incubated for 1 h at 37°C with 100 μl of hybridoma supernatants, diluted 1:2 in IPMA buffer (PBS with 0.66 M NaCl, 0.001 M Na_2EDTA, 1% [vol/vol] Tween 80, 0.1% NaN_3, and 5% specific-pathogen-free [SPF] calf serum). Plates were washed with PBS–0.5% Tween 20 and incubated for 1 h at 37°C with 100 μl of horseradish peroxidase (HRPO)-conjugated rabbit anti-mouse immunoglobulin (DAKO A/S; article no. P260; 1:1000 diluted in IPMA buffer without NaN_3) to detect bound MAbs. After washing, plates were developed with 3-amino-9-ethylcarbazole (Sigma; article no. A5754) and read microscopically. Noninfected EBTr monolayers were assayed simultaneously as negative controls.

IPMAs were also performed to evaluate the ability of a MAb to recognize different BHV1 strains. In these tests, separate EBTr monolayers were inoculated with different BHV1 isolates and incubated until CPE appeared. Thereafter, IPMA was performed as described above, using MAb from ascites.

RIPA. Polypeptide specificity of MAbs was determined by RIPA essentially as described by Westenbrink et al. (30). In short, confluent monolayers of EBTr cells were inoculated with BHV1 strain Lam. Radiolabelling was performed by adding, at 6 h postinfection (p.i.), L-[^{35}S]methionine (Amersham; 350 μCi/175-cm^2 tissue culture flask). Eighteen hours later, cells were washed and lysed in PBSTDS (PBS, 1% Triton X-100, 1.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS]). To 200 μl of cleared homogenate, 20 μl of ascitic fluid was added and incubated for 16 h at 4°C before addition of 100 μl of a 25% (wt/vol) suspension of protein A-Sepharose beads (CL-4B, article no. 17-0963-03; Pharmacia-LKB). After another 2 h at room temperature, the beads were washed with PBSTDS, after which immunocomplexes were solubilized in 50 μl of SDS sample buffer (14). Samples were analyzed on 10% polyacrylamide–SDS gels (14). After electrophoresis, gels were processed for fluorography, dried, and exposed to X-Omat films (Kodak) at −70°C (4, 15).

BHV1 isolates. A total number of 160 BHV1 isolates, both respiratory and genital strains, originating from 10 countries were incorporated in the study. Dutch isolates (n = 57) were obtained from the Regional Animal Health Services. Isolates from Belgium (n = 5) were kindly provided by G. Wellemans (Brussels), and isolates from Denmark (n = 2) were obtained from L. S. Christensen (Lindholm). S. Edwards (Weybridge, United Kingdom) supplied us with 2 isolates, and W. Strube (Leverkusen, Germany) gave us 30 isolates. Isolates from Spain (n = 2) and from Italy (n = 6) were obtained from E. Espuña (Gerona) and from G. Castrucci (Perugia), respectively. Twenty-three isolates originated from Hungary (A. Bartha, Budapest), and one originated from Canada (L. A. Babiuk, Saskatoon). M. Kennedy (Knoxville, Tenn.), S. M. Goyal (St. Paul, Minn.), and J. K. Collins (Fort Collins, Colo.) supplied us with 21 isolates from the United States. Finally, 11 vaccine strains were incorporated in our study.

Bovine serum samples. Negative field serum samples (n = 111) originated from Denmark (B. Nylin) and Switzerland (M. Ackermann), countries where vaccination never has been permitted and which are free from BHV1 infections. In addition, 93 serum samples were obtained from a Dutch farm with a negative history of BHV1 infections and screened serologically since 1984 (J. Sol). Positive (n = 161) field serum samples were kindly provided by A. Bartha (Hungary), G. Castrucci (Italy), E. Espuña (Spain), S. M. Goyal (United States), and M. Kennedy (United States).

In addition, 11 negative and 18 positive serum specimens which were used in a previous comparative study on 16 immunoassays for the detection of BHV1-specific antibodies (13) were incorporated in this investigation.

VNT. A 24-h virus neutralization test (VNT) was performed essentially as described by Bitsch (3). Serial twofold dilutions of test serum in Eagle's minimum essential medium with Earle's salts (EMEM) containing 2% SPF calf serum and antibiotics (100 IU of penicillin per ml, 110 μg of streptomycin per ml, 50 IU of nystatin per ml, and 100 μg of kanamycin per ml) were mixed with equal volumes of strain K22 of BHV1 (10) ($10^{3.3}$ $TCID_{50}$/ml) in the same medium. One hundred microliters of the mixtures was incubated at 37°C for 24 h in a CO_2 incubator before addition of 100 μl of a suspension of EBTr cells (250,000 cells per ml) in EMEM containing 10% SPF calf serum and antibiotics. After 3 days at 37°C in a CO_2 incubator, the EBTr cells were examined microscopically for CPE. The titer of the test serum was taken as the reciprocal of the highest dilution giving complete inhibition of CPE.

Blocking ELISA for BHV1-specific antibodies. (i) Reagents. Secondary bovine fetal kidney cells were grown in minimal essential medium with Hanks' salts supplemented with 10% SPF calf serum (free of bovine viral diarrhea virus' anti-bovine viral diarrhea virus antibodies, and anti-BHV1 antibodies) and with antibiotics (see above). Confluent monolayers were inoculated with a stock preparation of the Lam strain of BHV1. The maintenance medium consisted of EMEM supplemented with 2% horse serum and antibiotics (see above). When, 3 to 4 days after infection, extensive CPE was observed, cells and medium were frozen at −20°C. After thawing, the resulting cellular lysate was centrifuged at 1,000 × g (20 min). The supernatant was then centrifuged for 4 h at 8,500 × g, after which the virus-containing pellet was suspended in a small volume of PBS, cooled on ice, and disrupted with an ultrasonic disintegrator (MSE PG100 model 150W) at maximum power (three times, each for 15 s). Finally, the antigen preparation was clarified by centrifugation for 10 min at 800 × g and used at an optimal dilution to coat ELISA plates [see below, "(ii) Test procedure"]. Coating of the virus antigen was performed in 0.05 M carbonate buffer, pH 9.6. To each well of a 96-well ELISA plate (Greiner; article no. 655061), 100 μl of diluted antigen was added. Plates were sealed with tape, incubated at 37°C overnight, and stored at −20°C. Under these conditions, coated plates were stable for at least 1 year.

A MAb (CDI-BHV1-999.5.2.2), selected for its specificity to gB of BHV1, was conjugated with HRPO (Boehringer Mannheim; article no. 814407) according to the method described by Wilson and Nakane (32). The conjugate was dialyzed against PBS, after which glycerol was added to a final concentration of 50%. Storage was performed at −20°C. Before use, the conjugate was diluted at the appropriate concentration [see below, "(ii) Test procedure"] in ELISA buffer (0.01 M Na_2HPO_4, 0.5 M NaCl, 0.005 M KCl, 0.001 M Na_2EDTA, 0.05% [vol/vol] Tween 80, pH 7.3) containing 10% horse serum.

The substrate solution was freshly prepared by adding 55 mg of the chromogen 2,2′-azinobis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS; Sigma; article no. A-1888) and 50 μl of 30% H_2O_2 to 100 ml of 0.05 M citric acid buffer, pH 4.5.

(ii) Test procedure. The principle of the ELISA for measuring BHV1-specific antibodies is a blocking method in which the reaction of an epitope on gB with its corresponding MAb is blocked by specific antibodies that are present in the test sample. In the test procedure, all reagents were added in 100-μl quantities. After each incubation step, plates were washed six times with a solution of 0.05% (vol/vol) Tween 80 in

deionized water. The optimal dilution of the antigen preparation, to coat the ELISA plates, was chosen in such a manner that a near-maximum response was obtained in a checkerboard titration. The MAb-HRPO conjugate was used in the ELISA at such a dilution that the maximum optical density at 405 nm (OD_{405}) response, as obtained with fetal calf serum (FCS) (0% blocking), was between 1.0 and 2.0 after a substrate incubation of 1 to 2 h at room temperature.

Undiluted bovine serum or defatted milk samples were pipetted into the wells of previously washed antigen-coated ELISA plates. Then, each plate was sealed and incubated overnight at 37°C, after which the MAb-HRPO conjugate was added. After an incubation of 1 h at 37°C, substrate was added. Finally, after an incubation of 1 to 2 h at room temperature, color development was measured at 405 nm with a microplate reader (BIO-TEK Instruments Inc., Winooski, Vt.; model EL312).

The blocking percentage of each test sample was calculated by taking the OD_{405} of FCS as a reference (0% blocking) according to the following formula:

$$\frac{OD_{405}(\text{FCS}) - OD_{405}(\text{test sample})}{OD_{405}(\text{FCS})} \times 100$$

= blocking percentage of test sample

Serum ELISA titers were determined by testing serial twofold dilutions of test samples prepared in FCS. The titer was expressed as the reciprocal of the highest dilution still giving a blocking percentage of ≥50%.

RESULTS

We have performed two fusions (no. 998 and 999) with spleen cells of BHV1-immunized mice and obtained 41 BHV1-positive hybridoma clones. The specificity of the MAbs produced by these hybridomas was characterized by RIPA. Precipitated polypeptides were analyzed on SDS-polyacrylamide gels and compared with the polypeptides precipitated by characterized anti-BHV1 gB, anti-BHV1 gC, and anti-BHV1 gD MAbs (data not shown). Of these 41 anti-BHV1 MAbs, 15 have been identified as anti-BHV1 gB MAbs, 10 have been identified as anti-BHV1 gC MAbs, and 3 have been identified as anti-BHV1 gD MAbs. The specificity of the remaining MAbs could not be characterized with certainty. All MAbs are of the immunoglobulin G (IgG) isotype (isotype 1, 2a, or 2b) and have a kappa light chain. The anti-BHV1 gB MAbs precipitated polypeptides with a banding pattern characteristic for BHV1 gB: three prominent protein bands with apparent molecular weights of 120,000 to 130,000, 75,000, and 55,000, the most slowly migrating band being the least intense. These polypeptides comigrate with the polypeptides precipitated by the anti-BHV1 gB MAb 42/18/7 and match the three gB polypeptides observed by others (19, 28). The results of the RIPA of MAbs 12, 14, and 17 and of anti-BHV1 reference MAbs are shown in Fig. 1.

Ascitic fluids of five anti-BHV1 gB MAbs (MAbs 11, 12, 14, 17, and 18) have been further studied. MAb 14 [CDI-BHV1-999.5.2.2, isotype IgG2b(κ)] was chosen to be used in the ELISA, because its corresponding epitope gave rise to a more rapid and much higher primary antibody response after BHV1 infection than other epitopes on gB, gC, or gD. By using MAb 14 in three groups of four calves receiving different BHV1 strains (Lam, Iowa, or Harberink; 4×10^7 $TCID_{50}$ intranasally), antibodies were detected in most animals for the first time at day 8 p.i. and reached a maximum titer of at least 10^3 at day 13 p.i. (18).

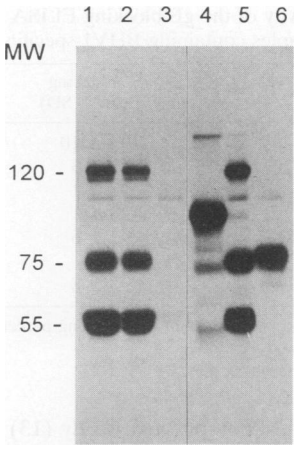

FIG. 1. RIPA MAbs 12, 14, and 17. Lysates of radiolabelled BHV1-infected cells were immunoprecipitated with MAb 12 (lane 1), MAb 14 (lane 2), MAb 17 (lane 3), anti-gC MAb 118/2/4 (lane 4), anti-gB MAb 42/18/7 (lane 5), and anti-gD MAb 21/3/3 (lane 6). The gel was prepared for autoradiography and exposed for 8 h. Weak bands in lane 3 became more intense after a prolonged exposure, resulting in the same banding pattern as that observed in lanes 1 and 2. The apparent molecular weights (MW) of the gB polypeptides have been indicated (in thousands) and were based on the ^{14}C-labelled marker CFA.626 of Amersham (not shown).

In order to ascertain that the epitope, as recognized by the selected MAb, represents a conserved structure on the BHV1 gB, as many as 149 BHV1 isolates, originating from 10 countries, and 11 vaccine strains were analyzed by IPMA. All EBTr monolayers, inoculated with each of the BHV1 isolates, showed a bright cytoplasmic staining pattern exclusively in the direct vicinity of the CPE. No IPMA staining was observed in noninfected EBTr monolayers. These results indicate that the recognized epitope on gB is highly conserved.

To determine the cutoff blocking percentage for positivity, bovine serum samples free from BHV1-specific antibodies (see Materials and Methods) were analyzed in the blocking ELISA. The ELISA results as obtained with 215 negative serum samples are given in Table 1. Taking the mean blocking percentage of all negative sera plus 2 standard deviations ($23 + 24 \approx 50\%$) as the cutoff value for positivity, the specificity of the test is 0.96 (206 of 215 samples).

The sensitivity of the test was determined with 161 positive field serum samples originating from four different countries (Table 2). In addition, 18 positive reference serum samples

TABLE 1. Specificity of the gB blocking ELISA as determined with antibody-negative serum samples

Origin of serum (*n*)	% Blocking (mean ± SD)	Specificity[a]
The Netherlands (93)	22 ± 12	0.97
Switzerland (15)	24 ± 16	0.93
Denmark (96)	26 ± 11	0.95
Reference sera[b] (11)	5 ± 8	1.00
Overall (215)	23 ± 12	0.96

[a] Samples are scored negative when the blocking percentage is <50%.
[b] See reference 13 and Table 3.

TABLE 2. Sensitivity of the gB blocking ELISA as determined with serum samples containing BHV1-specific antibodies

Origin of serum (*n*)	% Blocking (mean ± SD)	Sensitivity[a]
Italy (97)	93 ± 10	0.99
Spain (10)	91 ± 3	1.00
United States (44)	93 ± 1	1.00
Hungary (10)	93 ± 1	1.00
Reference sera[b] (18)	91 ± 6	1.00
Overall (179)	93 ± 8	0.99

[a] Samples are scored positive when the blocking percentage is ≥50%.
[b] See reference 13 and Table 3.

previously used in a comparative study (13) were also tested. Based on the results obtained with these 179 serum samples, an overall sensitivity of 0.99 (178 of 179 samples) was calculated. Only 1 of 179 serum samples scored negative and did not show any blocking activity in the test. Of the 178 positive serum samples, 167 showed a blocking percentage of ≥90%. The lowest positive response (74%) was observed with 1 of 18 reference serum samples. This serum specimen was taken from an SPF animal, 9 days after an intranasal inoculation with a field BHV1 isolate (Sp strain; Table 3).

To determine the detectability of the ELISA, twofold serial dilutions in FCS of a hyperimmune serum (serum P) (13) were assayed in the 24-h VNT as well as in the blocking ELISA. Neutralizing antibodies in serum P still could be detected by the VNT at a dilution of 1:2,048. The blocking ELISA was able to give a positive response (blocking percentage ≥50%) when serum P has been diluted up to 1:4,096 (Fig. 2).

By testing 40 reference serum specimens (13) in both the blocking ELISA and the 24-h VNT, a complete concordance was observed with defined positive and negative serum samples (Table 3). Exceptions were observed with serum samples obtained from seven nonvaccinated breeding bulls. These serum samples were previously found to respond positively in a highly sensitive indirect ELISA performed in Denmark by V. Bitsch (3a). Of these seven serum specimens, two responded in the 24-h VNT whereas five responded in the blocking ELISA (Table 3: AI serum samples originating from bulls of an artificial-insemination unit).

The ability of the blocking ELISA to detect antibodies shortly after infection was evaluated by testing sera which were obtained 7, 9, 11, 13, 15, and 17 days after animals had been inoculated intranasally with 10^5 $TCID_{50}$ of a BHV1 isolate (strain Cooper). The serum obtained 7 days after inoculation already gave a positive response in the blocking ELISA (percentage of blocking: 72%). In contrast, a commercially available indirect ELISA (test D) (13) and the 24-h VNT responded positively from day 11 (Fig. 3). The antibodies against gB in these animals remained detectable by the blocking ELISA until at least 3 years after infection (data not shown).

The blocking ELISA was also applied in an experimental setting by four routine diagnostic laboratories in The Netherlands, using test D (13) for routine diagnostic purposes. In test D, serum samples are diluted 1:25 before testing. Of 2,387 serum samples tested, 711 were scored positive by test D. All 711 serum samples except 7 gave also a positive response in the blocking ELISA. In contrast, 218 of the 1,676 serum samples that gave a negative response in the commercial test were positive in the gB blocking ELISA. Participating laboratories were asked to incorporate weak-positive control serum samples (blocking percentages: 65, 53, 56, and 64%) in each ELISA plate when performing the blocking ELISA. Overall, mean blocking percentages ± standard deviations of these control samples as measured by these laboratories were 65% ± 10% (n = 32), 56% ± 8% (n = 32), 58% ± 8% (n = 32), and 65% ± 7% (n = 30). The coefficient of variation (relative standard deviation) within each laboratory did not exceed 17%. These results indicate that the test, as performed by different laboratories, has a low and acceptable variance.

Finally, 100 individual milk samples and 95 tank milk samples were analyzed in the blocking ELISA as well as in the commercially available indirect ELISA (test D) (13). In both tests, milk samples were defatted by centrifugation and tested undiluted. As can be seen in Fig. 4, results of these samples show a high overall agreement in the two tests (192 of 195 = 0.98). Results of 195 samples are discordant in only three cases (1.5%).

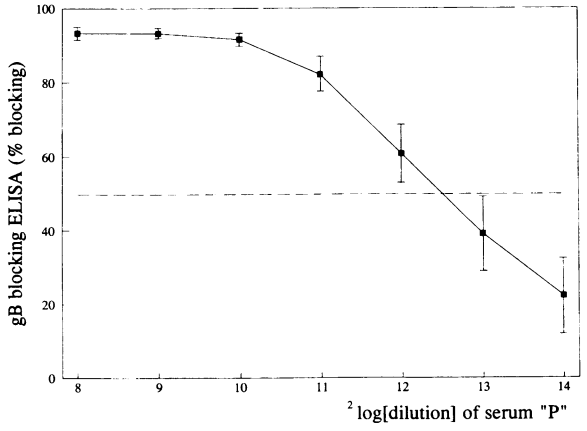

FIG. 2. Dose-response curve showing the relationship between a twofold serial dilution of a BHV1-specific hyperimmune serum (serum P) and the response in the gB blocking ELISA, expressed as blocking percentage. At each dilution of the serum, the interassay variation of 10 test runs is shown (mean ± standard deviation). A blocking percentage of 50% was taken as the cutoff value (dashed line).

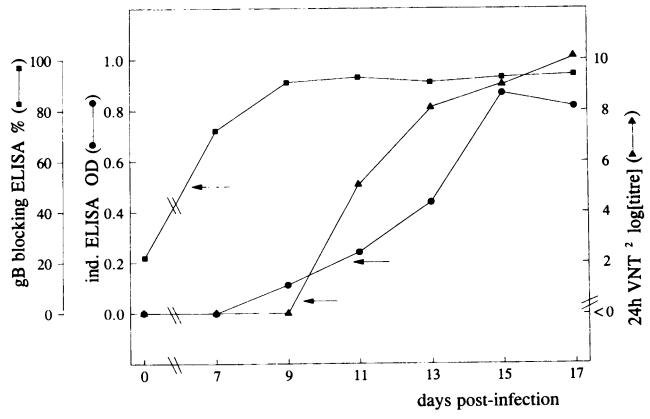

FIG. 3. Detection of BHV1-specific antibodies by three different tests in serum samples obtained shortly after intranasal infection. Arrows indicate cutoff values for positivity for each of three tests: for the gB blocking ELISA, cutoff value = 50%; for the commercially available indirect (ind.) ELISA, cutoff value = OD of 0.2; for the 24-h VNT, cutoff value, 2 log (titer) = 0.

TABLE 3. gB blocking ELISA results obtained with 40 reference serum samples

Serum[a]	Origin	% Blocking (blocking ELISA)[b]	Titer (24-h VNT)[c]
Negative (n = 11)			
Den 6	Serum from Denmark	6	<1
Den 7	Serum from Denmark	0	<1
Den 8	Serum from Denmark	2	<1
Den 9	Serum from Denmark	9	<1
Den 10	Serum from Denmark	0	<1
Vr 206	Farm with negative history	0	<1
Vr 213	Farm with negative history	8	<1
Vr 296	Farm with negative history	2	<1
Vr 326	Farm with negative history	27	<1
4797	BHV4 immunization	2	<1
4798	BHV4 immunization	1	<1
Positive (n = 18)			
F1	Field serum	94	64
F2	Field serum	95	128
F3	Field serum	95	256
F4	Field serum	94	16
F5	Field serum	94	64
628	Exptl infection	95	512
630	Exptl infection	95	256
632	Exptl infection	95	256
784	Exptl infection	94	128
P (1:16)	Hyperimmune serum	94	64
Lam 1	9 days p.i. (Lam strain)	86	2
Lam 2	13 days p.i. (Lam strain)	92	32
Sp 1	9 days p.i. (Spain strain)	74	1
Sp 2	13 days p.i. (Spain strain)	90	4
Har 1	9 days p.i. (Harberink strain)	80	1
Har 2	13 days p.i. (Harberink strain)	89	32
C 1	9 days p.i. (Cooper strain)	83	2
C 2	13 days p.i. (Cooper strain)	90	32
Unconfirmed, weakly positive and negative (n = 11)			
AI 1973	Nonvaccinated AI[d] breeding bull	32	<1
AI 5689	Nonvaccinated AI breeding bull	82	<1
AI 8912	Nonvaccinated AI breeding bull	91	<1
AI 9077	Nonvaccinated AI breeding bull	77	<1
AI Lurio	Nonvaccinated AI breeding bull	83	2
AI 02831	Nonvaccinated AI breeding bull	52	<1
AI 02966	Nonvaccinated AI breeding bull	24	2
2934	Tracherhine vaccinated	86	4
2936	Tracherhine vaccinated	83	1
H.B.1	High background	29	<1
H.B.2	High background	24	<1

[a] Origin of serum samples (13).
[b] Samples are scored positive when the blocking percentage is ≥50%.
[c] A titer of <1 is considered negative.
[d] AI, artificial insemination.

DISCUSSION

The gB of BHV1 is required for the penetration of the virus into cells and is essential for virus infectivity (25). The protein therefore will never be absent in wild-type virus strains. Moreover, the ability of the selected MAb to recognize all of 160 different BHV1 strains indicates that the recognized epitope on gB is conserved and will be present in most, if not all, BHV1 field strains.

The reliability of the ELISA (specificity and sensitivity) was determined by testing 215 well-defined negative and 179 well-defined positive serum samples originating from several countries (Tables 1 and 2). The high sensitivity of the blocking test, which is able to identify 178 of 179 anti-BHV1 positive serum specimens originating from five different countries, indicates that the gB-specific MAb as used in the test recognizes an epitope located on gB of BHV1 that induces in most animals an antibody response.

Because gBs of alphaherpesviruses, including herpes simplex virus, pseudorabies virus, and BHV1, show a strong sequence homology (20, 25), the possibility that sera containing antibodies directed to alphaherpesviruses other than BHV1 give positive responses in the blocking ELISA due to immunologic cross-reactivity cannot be excluded. However, sera containing antibodies directed against herpesviruses other than BHV1 were not incorporated in our study.

When serially diluted serum samples were tested in both the blocking ELISA and the 24-h VNT (3), it was observed that the ELISA was able to detect lower antibody levels than the 24-h VNT. In addition, seroconversion after experimental infection was detected earlier by the blocking ELISA than by the 24-h

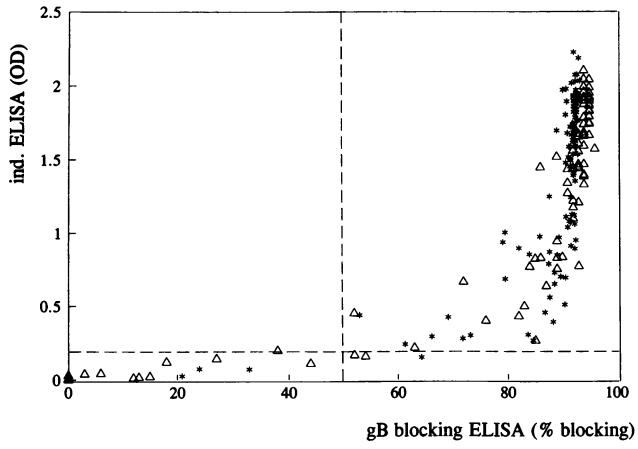

FIG. 4. Relationship between results of the gB blocking ELISA and of a commercially available indirect (ind.) ELISA as obtained with undiluted milk samples (*, individual samples, $n = 100$; △, tank milk samples, $n = 95$). Dashed lines represent cutoff values for positivity.

VNT (Fig. 3). A maximum response, expressed as blocking percentage, can be found already at day 9 p.i. (Fig. 3) when high levels of early IgM antibodies and no or minimal amounts of IgG antibodies are present (27).

In this study, we used serum samples from seven nonvaccinated bulls (Table 3). In these sera low antibody levels were detected by an indirect ELISA adapted from the one used in Denmark (1, 3a, 13). In a comparative study on 16 ELISAs, this indirect ELISA showed a high specificity and the highest sensitivity. Results with the seven bull serum samples obtained with the blocking ELISA were very similar to those obtained with the sensitive indirect ELISA from Denmark. Of the seven serum samples, five were found positive and two were negative by the blocking test. The indirect ELISA from Denmark scored these five and two serum samples as positive and doubtful, respectively (13). The 24-h VNT scored only two of seven serum samples positive (Table 3). However, it has not been clarified whether these bulls were latent carriers of BHV1. It is therefore impossible to conclude whether these results were true or false positives.

In a recently performed European sample exchange program, a comparison of serological diagnostic tests for BHV1 infection was made by dispatching 65 serum samples to nine laboratories in eight European countries (23). In this comparative study, the blocking ELISA described here showed the highest sensitivity. All positive ($n = 17$) and unconfirmed or weakly positive serum samples ($n = 13$) gave a positive response. In contrast, 1 of 19 negative serum samples gave a positive response. The high sensitivity of the blocking ELISA was also observed in this exchange program by testing two sets of serial dilutions of known positive samples (a serum sample diluted 1:16 to 1:1,024 and a sample containing purified specific IgG diluted 1:200 to 1:102,400) (23). In this study, the gB blocking ELISA revealed the highest titers (1,024 and 3,200, respectively).

The results obtained by four routine laboratories with 2,387 serum samples strongly suggest that the gB blocking test detects low antibody levels, present in at least some of the sera, which remain undetectable by a relatively reliable test (test D) (13) selected from several commercially available ELISA kits. Moreover, the results obtained by these laboratories with weakly positive serum samples, incorporated in each test run as internal controls, indicate that the blocking test can be performed with low variance. Preliminary results (unpublished), obtained by interlaboratory tests performed as described by the International Organization for Standardization (split-level experiment) (9) revealed a relative repeatability and reproducibility of test results (antibody serum titers ranging between 25 and 500) of approximately 15 and 25%, respectively (95% probability level).

Among the different serological tests able to detect BHV1-specific antibodies at low level, a competitive (blocking) ELISA, in which undiluted serum can be assayed without evidence of causing high background or nonspecific blocking, seems to be the test system of preference (23, 24).

On the basis of the close agreement between the commercially available indirect ELISA (test D) and the gB blocking test when undiluted milk samples are tested (Fig. 4), it is likely that these tests are suitable for antibody detection in this type of specimen. However, tests with paired serum-milk samples should be carried out to determine the reliability of each test when performed with milk samples.

To minimize the risk of introducing an infected animal into a BHV1-free artificial insemination center or herd, serological tests should be as sensitive as possible to avoid false-negative results when low anti-BHV1 antibody levels are present. A low antibody response in animals after BHV1 infection may occur when cattle become infected via the genital tract (6, 8, 12, 21). In addition, low serum antibody levels may be present in animals that became infected when maternal antibodies were present, as has been found after bovine respiratory syncytial virus infections (11). Therefore, the weakly positive ELISA responses as observed with the serum samples of the seven breeding bulls indeed may reflect infected animals (latent carriers) (13).

In conclusion, the gB blocking ELISA described in this paper is a simple, convenient, specific, and highly sensitive assay for the detection of BHV1-specific antibodies in serum. The sensitivity of this test appears to be superior to that of other serological diagnostic procedures, including 24-h VNTs and commercially available ELISAs. To minimize any risk of introducing latent BHV1 carriers among noninfected animals, the gB blocking test therefore would be the test of preference.

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