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Use of Epitope Mapping To Identify a PCR Template for Protein Amplification and Detection by Enzyme-Linked Immunosorbent Assay of Bovine Herpesvirus Type 1 Glycoprotein D

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Infection with bovine herpesvirus type 1 (BHV-1) occurs worldwide and causes serious economic losses due to the deaths of animals, abortions, decreased milk production, and loss of body weight. BHV-1 is frequently found in bovine semen and is transmitted through natural service and artificial insemination. The detection of BHV-1 in bovine semen is a long-standing problem in veterinary virology which is important in disease control schemes. In the present study, ordered deletions of the full-length BHV-1 glycoprotein open reading frame were used to identify an epitope recognized by a specific monoclonal antibody (MAb). A glycoprotein D fragment containing this epitope was then amplified using an in vitro protein amplification assay developed previously (J. Zhou, J. Lyaku, R. A. Fredrickson, and F. S. Kibenge, J. Virol. Methods 79:181-189, 1999), and the resulting peptide was detected by indirect enzyme-linked immunosorbent assay (ELISA) with the specific MAb. This method detected 0.0395 50% tissue culture infective dose of BHV-1 in raw bovine semen, which was 1,000-fold more sensitive than traditional PCR. We therefore conclude that this in vitro protein amplification assay combined with ELISA has superior sensitivity for direct virus detection in clinical samples.

Bovine herpesvirus type 1 (BHV-1) is responsible for a variety of diseases in cattle, including respiratory and genital infections, conjunctivitis, abortion, and enteritis, causing great economic loss to the cattle industry worldwide (6). As in other alphaherpesviruses, BHV-1 glycoproteins are the major structural components of the viral envelope and virus-infected cell membranes. The glycoproteins play important roles in virus-cell interactions, including recognition and attachment of the virion and its penetration into susceptible cells (8, 10, 12), viral neutralization, and immune destruction of infected cells (7, 13). Glycoprotein D (gD) of BHV-1, a homologue of herpes simplex virus gD, is one of the four essential major glycoproteins, together with gB, gC, and gH, which have been identified on the virus envelope and the plasma membrane of BHV-1 infected cells (22). It stimulates a potent neutralizing antibody response in animals and induces significant protection against BHV-1-induced diseases. Moreover, BHV-1 gD is a very stable antigen whose epitopes do not change under selective pressure (18).

It has also been reported that monoclonal antibodies (MAbs) against gD show the highest complement-independent virus-neutralizing activity and inhibit virus adsorption and penetration (4, 9, 23). MAbs against BHV-1 gD and their use in diagnostic tests, epitope mapping, and functional analysis have been reported by several investigators, including van Drunen et al. (22), Marshall et al. (15, 16), Hughes et al. (9), Dubuisson et al. (4), Abdelmagid et al. (1, 2), and Shen et al. (18). These functional attributes of gD make it one of the most important viral proteins and an excellent target viral antigen for the detection of BHV-1 in clinical samples, including nasal or vaginal secretions and semen.

BHV-1 is frequently found in bovine semen and can be widely transmitted through artificial insemination. The detection of BHV-1 in bovine semen is a long-standing problem in veterinary virology which is important in disease control schemes. None of the methods developed so far have been found wholly satisfactory for general use in diagnostic laboratories, especially when applied to semen donor bulls (29). Under these circumstances, our laboratory developed a new method of virus detection consisting of a protein amplification assay following PCR of the BHV-1 gD gene (29). As the gD polypeptide obtained in the protein amplification assay resembles the gD expressed in *Escherichia coli* in the absence of glycosylation, a panel of MAbs specific to *E. coli*-expressed gD was also raised (14). In this study, we report the use of an enzyme-linked immunosorbent assay (ELISA) technique to detect gD with specific MAbs and the identification of an epitope between amino acid positions 216 and 417 of gD recognized by a group of MAbs. Studies are also described targeting this epitope in the protein amplification assay along with detection of the resulting polypeptide by ELISA with the selected MAbs to develop a simple, rapid, sensitive, and more reliable method of direct virus detection.

MATERIALS AND METHODS

Virus strains and cell culture. The Los Angeles (LA) strain of BHV-1 was obtained from the American Type Culture Collection (Manassas, Va.). Madin-

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Darby bovine kidney (MDBK) cells were grown in minimum essential medium (GIBCO Life Technologies) containing 100 U of penicillin/ml, 100 μg of streptomycin/ml, 25 μg of fungizone (Invitrogen Life Technologies)/ml, and 10% fetal bovine serum and maintained in 75-cm^2 flasks in minimum essential medium with Earle's salts with the same concentration of antibiotics and the fetal bovine serum reduced to 2%. The flasks were incubated at 37°C with a 5% CO_2 air atmosphere, and the cell monolayers were infected the next day with virus diluted 1:10. The virus was harvested after 3 to 4 days and purified on a Ficoll 400 (Amersham Pharmacia Biotech) step gradient (25:10) after precipitation with ammonium sulfate. The protein concentration in the purified virus was estimated by spectrophotometry at 280 nm prior to storage at −80°C.

BHV-1-infected cell lysate. MDBK cells infected with the LA strain of BHV-1 were used 16 h postinfection. They were lysed with 0.5% Nonidet P-40 (NP-40) (Sigma Chemicals, St. Louis, Mo.) and clarified. Uninfected cell lysate was similarly prepared, and the protein concentration was estimated and samples were stored as for the purified virus.

In vitro spiking of semen with BHV-1. Semen samples collected from a bull and stored at −80°C were diluted 1:20 to a final volume of 90 μl using PCR buffer (50 mM KCl, 10 mM Tris-HCl [pH 8.3], 2.5 mM $MgCl_2$, 0.1 mg of gelatin/ml, 0.45% NP-40, and 0.45% Tween 20). Tenfold serial dilutions of BHV-1 strain LA stock culture containing $10^{5.5}$ 50% tissue culture infective doses ($TCID_{50}$)/100 μl were made in the PCR buffer, and 10 μl of each dilution from 10^{-1} to 10^{-6} was added to the diluted semen.

Construction of pGEX expression plasmids and preparation of fusion proteins. The BL21(DE3)(pLysS) strain of *E. coli* transformed with pGEX plasmids containing the entire open reading frame (ORF) of the gD gene (14) was used to express the full-length glutathione *S*-transferase (GST)-gD fusion protein. In order to obtain overlapping fragments of the gD ORF, three forward primers designated A, C, and E and two reverse primers designated B and D, based on the published nucleotide sequence of the gD gene of the P8-2 strain of BHV-1 from nucleotide positions 77 to 1341 (20), were used in the PCR. The forward primers were designed to contain a *Bam*HI restriction site (italic) with primer A located from nucleotide positions 84 to 98 (5′ *GGA TCC* ATG CAA GGG CCG AC 3′), primer C located from nucleotide positions 730 to 744 (5′ *GGA TCC* GCC CGG GAT TAC GA 3′), and primer E located from nucleotide positions 412 to 425 (5′ *GGA TCC* ATC GAG AGC CGG TG 3′). The reverse primers were designed to contain the Streptag sequence (lowercase) (19) and *Xho*I restriction site (italic) with primer B located from nucleotide positions 997 to 1013 (5′ *CTC GAG* tca acc gaa ctg cgg gtg acg cca agc gct CC GTC GCC TTC GGG TCC 3′) and primer D located from nucleotide positions 1315 to 1335 (5′ *CTC GAG* tca acc gaa ctg cgg gtg acg cca agc gct CCC GGG CAG CGC GCT GTA GTT 3′). For the in vitro transcription and translation reactions, forward primer C was designed to contain T7 RNA polymerase promoter sequence with an ATG codon (5′ GTA AAA CGA CGG CCA GTG AAT TGT AAT ACG ACT CAC TAT AG GG ATG GCC CGG GAT TAC GA 3′) and the reverse primer D was designed without the Streptag sequence and *Xho*I restriction enzyme site (5′ TCA CCC GGG CAG CGC GCT GTA GTT 3′) (29). The BHV-1 DNA preparation and PCR procedure used to obtain specific amplification of the gD gene DNA were described previously (29). The PCR products were purified from low-melting-point agarose gels (5) and then cloned into the TA cloning vector pCRII (Invitrogen Life Technologies). The inserts were subcloned into the pGEX-5X-3 expression vector, which was then used to transform competent *E. coli* strain BL21(DE3)(pLysS). The transformed *E. coli* cells were grown overnight in 2× YT (yeast and tryptone) medium (Becton Dickinson and Co., Paramus, N.J.) supplemented with 0.1 ml of 5-mg/ml ampicillin, 5 μl of 35-μg/ml chloramphenicol (Roche Molecular Biochemicals), and 0.25 ml of 40% glucose. Protein expression was induced by adding IPTG (isopropyl-β-D-thiogalactoside) (Amersahm Pharmacia Biotech) to a final concentration of 0.1 mM. The GST-gD fusion proteins were purified by affinity chromatography using a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech) according to the manufacturer's instructions. The purified GST-gD fusion proteins were concentrated using centricon (Millipore Corp., Bedford, Mass.). The concentrations of the purified proteins were estimated by spectrophotometry at 280 nm, and their purity and integrity were analyzed by resolving them on discontinuous sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12.5% PAGE) (11) and visualizing them with Coomassie blue R-250 (Bio-Rad) and, if necessary, by staining them with a silver staining kit (Bio-Rad). The BL21 strain of *E. coli* without a gD gene was lysed with 0.5% NP-40 (Sigma) and then clarified at 700 × *g* to obtain the bacterial lysate without gD.

For the amplification of the CD fragment of the gD ORF from semen samples infected with BHV-1, the semen samples were digested with proteinase K for 60 min at 55°C and then heated for 10 min to denature the proteinase K and clarified at 13,000 × *g* for 30 min, and 2.5 μl of the supernatant was directly used for PCR (29). PCR with a DNA template prepared from the purified BHV-1 was used as a positive control.

GST-gD-specific MAbs. The GST-gD-specific hybridoma cells of clones 26, 12, 4F7, 5D4, 2D7, 2C4, and 10B5, developed and characterized previously (14), were used to raise mouse ascites fluid. For each clone, this was done by intraperitoneal inoculation of 10^7 hybridoma cells into BALB/c mice that had been primed 2 to 3 weeks previously with 0.5 ml of pristane (Sigma). The clarified ascites fluid containing immunoglobulin M (IgM) MAbs were used as is, and IgG MAbs were purified by affinity chromatography using protein G columns (17) and were then concentrated using polyvinyl pyrrolidone (Sigma).

Western blotting. Purified GST-gD and BHV-1 proteins were transferred from the SDS-PAGE gel to the nitrocellulose membrane and then blocked with 3% skim milk. The membrane was then reacted with a 1:100 dilution of the MAb. Subsequent steps were performed with protein A protein L-horseradish peroxidase conjugate (Clontech Laboratories) and a peroxidase substrate kit (Bio-Rad) according to the directions of the manufacturers.

ELISA. The full-length GST-gD fusion protein and the MAbs were used to develop the ELISA, in which purified BHV-1 and infected cell lysate were used as positive controls and uninfected cell lysate and bacterial lysate without gD served as negative controls. To determine the optimum volumes and concentrations of the reagents, preliminary checkerboard titration experiments were conducted with all the antigens and MAbs. The best results were obtained when 10 μg of GST-gD fusion protein and 20 μg of purified BHV-1 or infected cell lysate were used to coat each well of the plate and were reacted with 1:100 dilution of the MAbs. For the indirect ELISA, the antigens were diluted in 0.2 M bicarbonate buffer, pH 9.6, and 100 μl was used to coat each well of a 96-well Immulon II microtiter immunoassay plate (Dynatech Laboratories, Chantilly, Va.). The plates were incubated overnight at 4°C. The antigens were removed, and the plates were blocked with 100 μl of 5% horse serum diluted in 1× Dulbecco's phosphate-buffered saline (Invitrogen Life Technologies) containing 0.05% Tween 20 and incubated at room temperature for 1 h. The plates were then treated with 100 μl of a 1:100 dilution of MAbs/well and incubated at room temperature for 2 h. This was followed by incubation with 100 μl of a 1:30,000 dilution of alkaline phosphatase-labeled goat anti-mouse IgM-IgG (Sigma)/well for 1 h at room temperature. The plates were reacted with the substrate *p*-nitrophenyl phosphate (Bio-Rad) until the color development was complete. The optical density was measured at 405 to 490 nm ($OD_{405-490}$) using a Spectra MAX 340 (Molecular Devices Corp., Sunnyvale, Calif.) ELISA reader. The steps were separated by extensive washing with 1× Dulbecco's phosphate-buffered saline containing 0.05% Tween 20. All the experiments were done in triplicate, and the mean OD was calculated. The ODs of the positive samples were expressed after subtracting the OD of the corresponding negative control. An $OD_{405-490}$ cutoff value for the ELISA to differentiate positive from negative samples was set at 2 standard deviations above the average $OD_{405-490}$ of the negative antigens. Values above this cutoff were scored positive (3). For the detection of CD polypeptide in the in vitro transcription-translation reaction, 96-well microtiter plates were coated with 50 μl of the TNT reaction mixture diluted to 100 μl in 0.2 M bicarbonate coating buffer, and ELISA was carried out as described above using MAb 26. In vitro transcription and translation reactions without PCR products served as negative controls, and the *E. coli*-expressed CD fragment of gD served as the positive control.

Epitope mapping. MAbs 2D7 (IgG), 26, 12, 4F7, and 5D4 (IgM) were reacted individually with full-length GST-gD and the overlapping protein fragments AB, CD, and ED in indirect ELISA to identify their epitopes on recombinant proteins. Bacterial lysate without gD was used as the negative control. Epitopes of the MAbs were located based on the reactivities of the MAbs with individual protein fragments and the full-length GST-gD. OD readings equal to or above the OD readings of full-length GST-gD were considered positive. OD readings below the OD readings of full-length GST-gD but above the cutoff value were considered partial reactions. The percentages of reactions of various fragments with different MAbs in indirect ELISA were also calculated by considering the reaction of the full-length GST-gD as 100%.

Preparation of CD protein fragment of gD from PCR products. CD peptides from PCR products were prepared as described previously (29) using the TNT T7-coupled reticulocyte lysate system (TNT-RLS; Promega). Briefly, 2.5 μl of the PCR products of the CD fragment from purified BHV-1 DNA with the forward primer containing the T7 promoter sequence (29) were used directly in an in vitro transcription-translation mix containing 40 μCi of [^{35}S]methionine (Amersham Pharmacia Biotech) in 50-μl reaction volumes, using the TNT-RLS according to the manufacturer's specifications. A luciferase DNA (supplied by Promega) was used as a positive control. The reaction products were analyzed for size by SDS-PAGE and autoradiography. Nonradioactive TNT-RLS reactions with cold methionine were set up with the PCR products of the CD fragment of

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TABLE 1. ELISAs for three different BHV-1 gD antigens

MAb	Isotype	$OD_{405-490}$[a]		
		GST-gD	Purified BHV-1	Infected cell lysate
2D7	IgG2b	0.869 ± 0.09	0.110 ± 0.008	0.112 ± 0.008
2C4	IgG2b	1.632 ± 0.09	0.503 ± 0.08	0.000
10B5	IgG3	0.378 ± 0.01	0.638 ± 0.05	0.094 ± 0.003
26	IgM	0.438 ± 0.04	0.638 ± 0.05	0.015 ± 0.003
12	IgM	0.289 ± 0.02	0.369 ± 0.009	0.170 ± 0.02
4F7	IgM	0.129 ± 0.007	0.289 ± 0.07	0.144 ± 0.02
5D4	IgM	0.336 ± 0.02	0.193 ± 0.01	0.057 ± 0.006

[a] Mean OD readings (± the standard error) of three replicates after subtracting the mean OD readings of the negative control.

the gD ORF from purified BHV-1 DNA and infected semen samples. The CD peptide was then detected by indirect ELISA.

RESULTS

Analysis of various BHV-1 gD antigens by MAbs. ELISA was used to determine the antigenic characteristics of GST-gD, purified BHV-1, and infected cell lysate and to confirm the presence of specific epitopes. All the MAbs reacted specifically with the purified GST-gD, purified BHV-1, and infected cell lysate. The results are summarized in Table 1. The reactions of MAbs 26 and 5D4 with the infected cell lysate were weaker than the reactions of the rest of the MAbs, and MAb 2C4 did not react at all with the infected cell lysate. The purified IgG MAb 2D7 gave a positive reaction with a concentration of GST-gD as low as 0.312 μg/well, whereas the IgM MAbs were not able to detect concentrations below 1.25 μg/well. However, all MAbs failed to react with GST-gD and purified BHV-1 in Western blotting except MAb 26, which showed a weak but specific reaction with purified BHV-1.

DNA amplification of gD gene. PCR using primers A and B gave a PCR product of 971 bp, primers C and D gave a PCR product of 648 bp, primers E and D gave a PCR product of 966 bp, and primers A and D gave the full-length gD gene PCR product of 1,293 bp shown in Fig. 1.

Protein expression and SDS-PAGE analysis. Expression of BHV-1 gD and the overlapping fragments AB, CD, and ED (designated according to the primers used to amplify their sequences) with GST tags allowed the purification of the fusion proteins by affinity chromatography on glutathione-Sepharose 4B columns. Figure 2 shows the *E. coli*-expressed GST-gD fusion protein and overlapping protein fragments analyzed by SDS-PAGE. Separation of GST from the fusion proteins occurred during SDS-PAGE. The amino acid sequences expressed by the various protein fragments are represented schematically in Fig. 3.

Epitope mapping. In the epitope-mapping experiments, all the MAbs reacted specifically with full-length GST-gD and protein fragments CD and ED, suggesting that their epitope lies between amino acid positions 216 and 417, i.e., on the protein fragment CD. The results are summarized in Table 2. A partial reaction ranging from 25 to 66% of the reaction to the full-length protein was observed with protein fragment AB.

Detection of BHV-1 in virus-infected semen by protein amplification-ELISA. In vitro transcription-translation of the CD fragment PCR product yielded only one protein band with

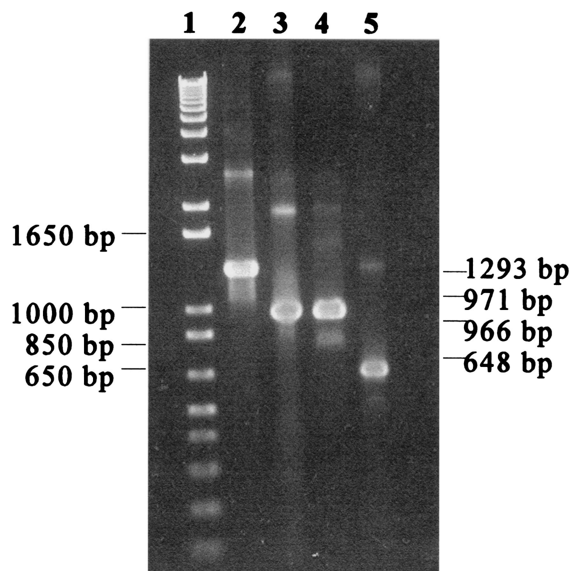

FIG. 1. PCR amplification of gD gene DNA and overlapping fragments of gD ORF. Lane 1, 1-kbp-plus DNA ladder; lane 2, gD gene DNA; lanes 3, 4, and 5, overlapping fragments AB, ED, and CD, respectively.

approximately the same size as that of the CD fragment expressed in *E. coli* (Fig. 4). In ELISA, MAb 26 reacted specifically with the CD polypeptide in the reactions where PCR products were amplified from purified BHV-1 and from semen infected with virus dilutions of 10^{-1} to 10^{-4}. The comparison of PCR and protein amplification-ELISA for the detection of BHV-1 in bovine semen is summarized in Table 3. PCR could detect the virus only in semen infected with 10^{-1} virus sample

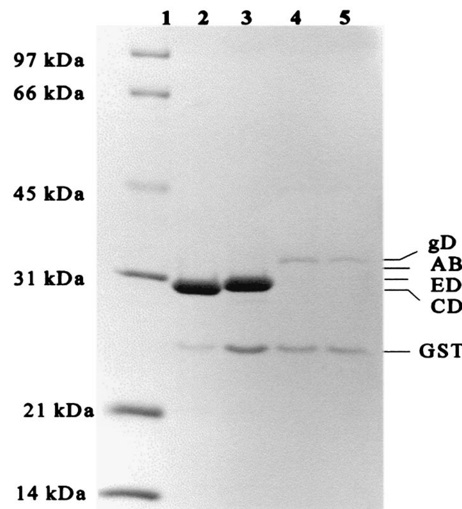

FIG. 2. SDS-PAGE analysis of affinity-purified GST-gD and various overlapping protein fragments. Lane 1, protein standards (Bio-Rad), with values indicated on the left; lanes 2, 3, and 4, overlapping protein fragments CD, ED, and AB, respectively; lane 5, full-length gD. In each case, the GST fragment has been cleaved away (as discussed in the text) and is indicated at the low-molecular-weight end of the gel. The positions of gD, CD, ED, AB, and GST are indicated on the right.

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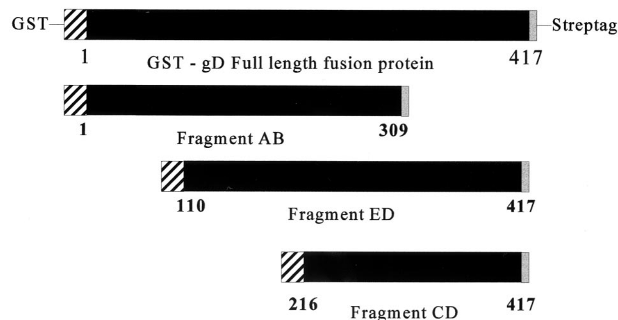

FIG. 3. Schematic representation of BH-1 gD and its segments that were expressed by *E. coli* as GST fusion proteins. Each protein is fused with a GST tag at the amino end and Streptag at the carboxyl end.

having a virus titer of 790 $TCID_{50}$/50 μl (Fig. 5), whereas protein amplification-ELISA detected BHV-1 in semen seeded with 10^{-4} virus sample with a virus titer of 0.79 $TCID_{50}$/50 μl. Thus, even without considering the different amounts of samples used in the two methods, there is a direct observation of a 1,000-fold increase in sensitivity by protein amplification-ELISA over traditional PCR.

DISCUSSION

gD of BHV-1 is one of the major and best-characterized envelope proteins of the virus, which possess epitopes that do not change under selective pressure (18). Several diagnostic tests have been developed targeting the gD gene (24, 25, 26, 27), including a new method of virus detection consisting of a protein amplification assay following PCR amplification of the BHV-1 gD gene (29). It has been shown that MAbs specific for gD expressed in *E. coli* can be used to detect the gD polypeptide produced by protein amplification assay in ELISA, since the *E. coli*-expressed BHV-1 gD fusion protein is similar to the unglycosylated gD made by the protein amplification assay (14). In this study, we have developed an ELISA technique to detect a GST-gD fusion protein with specific MAbs and identified their epitopes between amino acid positions 216 and 417 of BHV-1 gD. The identified epitopes were then targeted in the protein amplification assay, and the resulting polypeptide was detected by MAbs in ELISA.

The conditions for ELISA were optimized in order to detect GST-gD using specific MAbs. Specific reaction of the MAbs with purified BHV-1 and infected cell lysate confirmed the specificities of the MAbs for gD epitopes. The reduced activities of MAbs 26 and 5D4 and the lack of activity of MAb 2C4 with infected cell lysate might be due to the partial loss of

TABLE 2. ELISA reactivities of MAbs with recombinant proteins

E. coli-expressed GST-gD amino acid sequences	MAb reactivity[a]				
	2D7	26	12	4F7	5D4
1–417 (GST-gD)	+	+	+	+	+
1–309 (AB)	+/−	+/−	+/−	+/−	+/−
110–417 (ED)	+	+	+	+	+
216–417 (CD)	+	+	+	+	+

[a] +, positive reaction; +/−, partial reaction (see the text for details).

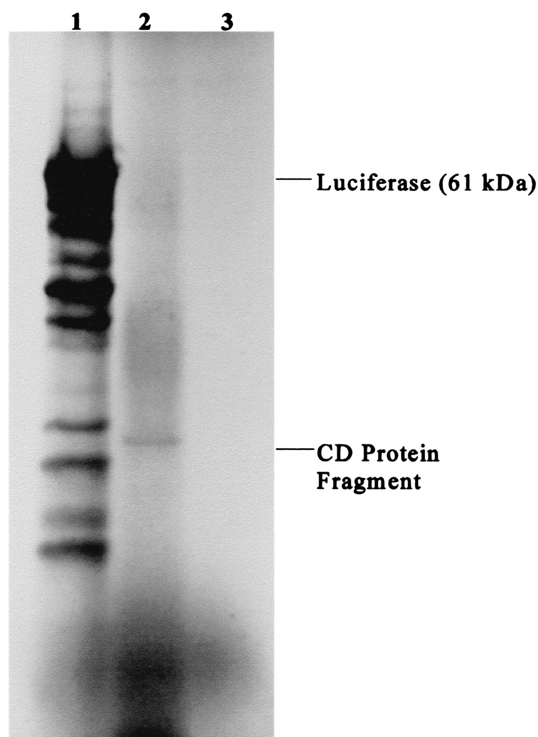

FIG. 4. Detection of the CD peptide of gD by autoradiography after in vitro transcription and translation. Lane 1, luciferase control reaction product at 61 kDa full length; lane 2, CD peptide; lane 3, in vitro transcription and translation reaction product without the template DNA.

antigenicity as a result of treatment with NP-40 (28). Alternatively, it could be due to a lower protein concentration in the antigen preparation. However, all the MAbs failed to react with GST-gD in Western blot analysis. It has been observed that the GST portion of the fusion proteins separates from the fusion partner during SDS-PAGE (14). Separation of the GST portion and/or the reducing conditions of SDS-PAGE might have caused variation in the conformational properties of the epitopes with respect to the complete GST fusion proteins, thus reducing antibody binding in the Western blots.

Several approaches have been used to detect antigenic sites

TABLE 3. Comparison of PCR and protein amplification-ELISA for the detection of BHV-1 in bovine semen

Source of PCR template	Dilution of virus stock	PCR result using primers C and D[a]	Protein amplification-ELISA $OD_{405–490}$[b]
Purified BHV-1		+	0.206 ± 0.15
Infected semen	10^{-1}	+	0.293 ± 0.09
Infected semen	10^{-2}	−	0.461 ± 0.12
Infected semen	10^{-3}	−	0.513 ± 0.08
Infected semen	10^{-4}	−	0.220 ± 0.10
Infected semen	10^{-5}	−	0.000
Infected semen	10^{-6}	−	0.000

[a] +, positive PCR result with a product of 648 bp seen on agarose gel; −, negative PCR result with no product seen on agarose gel.
[b] Mean OD readings (± the standard error of the mean) of three replicates after subtracting the mean OD of the negative control.

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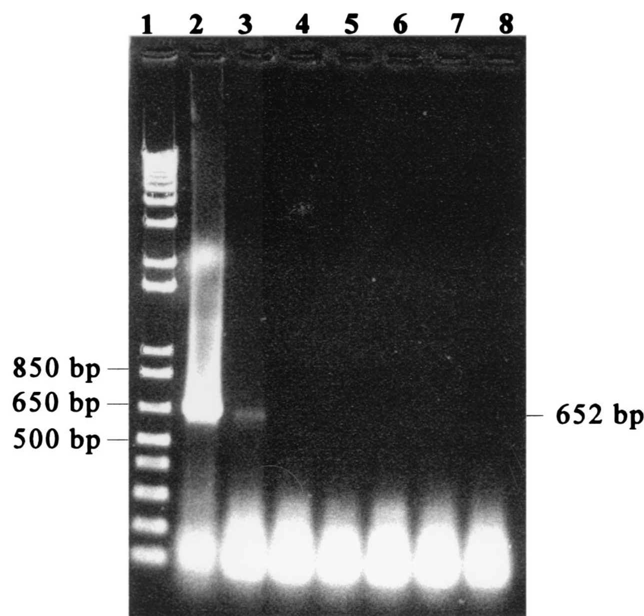

FIG. 5. PCR amplification of CD fragment of gD gene using purified BHV-1 DNA and infected semen samples. Lane 1, 1-kbp-plus DNA ladder (Invitrogen Life Technologies); lane 2, PCR product from purified BHV-1 DNA; lanes 3 to 8, PCR products from semen samples infected with virus dilutions from 10^{-1} to 10^{-6}, respectively.

on BHV-1 gD, including competition binding assays (9, 15, 22) and expression and analysis of mutant forms of gD in various expression systems, such as the mammalian expression system (21), *E. coli* (1), and the baculovirus-insect cell system (2). In this study, epitope mapping was carried out using three overlapping fragments of gD expressed in *E. coli*. Based on the reactions of the MAbs with protein fragments in ELISA, their epitope(s) has been located on fragment CD, i.e., between amino acid positions 216 and 417 of gD. A partial reaction ranging from 25 to 66% of the reaction to the full-length protein observed with protein fragment AB suggests that a portion of the epitope of these MAbs lies on fragment AB. The exact nature of the identified epitope can only be determined after more ordered deletions on the CD protein fragment. The identified epitope may be a linear one for the following reasons. (i) It has already been shown that only linear epitopes are preserved in *E. coli*-expressed gD of BHV-1 because of a lack of posttranslational modifications in *E. coli* (1). (ii) All the cysteine residues that could possibly contribute to disulfide bonding and tertiary-structure formation are located between amino acid positions 74 and 214 (21). The protein fragment CD, which contains the epitope, begins at amino acid position 216, thus reducing the possibility of formation of conformational epitopes.

During BHV-1 infection, semen is most likely contaminated at the time of ejaculation by virus particles shed from the infected mucosa of the prepuce, the penis, and possibly the distal part of the urethra. Therefore, the virus in bovine semen is present mostly in the seminal fluid fraction but also in the nonsperm cells and adsorbed to spermatozoa (24). There are no significant differences in the distribution of virus within the semen between artificially contaminated bovine semen and bovine semen infected naturally (28). Thus, using the artificially infected bovine semen, it is possible to accurately determine the sensitivity of detection of BHV-1 in bovine semen (28, 29). In order to improve the sensitivity of the protein amplification assay (29) and to make it simpler and more rapid, the CD fragment of the gD gene was targeted in the PCR, followed by an in vitro transcription-translation reaction using a TNT lysate system. The presence of a single polypeptide of appropriate molecular mass (Fig. 4) confirmed that only the CD fragment of the gD gene had been transcribed and translated in the TNT reaction. MAb 26 was selected for the detection of the CD peptide resulting from the TNT reaction because of its performance in the epitope-mapping experiments. This MAb reacted specifically with the CD peptide obtained from protein amplification of purified BHV-1 DNA and of infected semen up to a virus dilution of 10^{-4}. The differences in the OD readings (Table 3) may be a reflection of the variation in the amount of DNA present in the volume of the PCR product that was used for TNT reactions. The traditional PCR detected the virus only in bovine semen infected with a 10^{-1} virus sample and having a titer of 790 $TCID_{50}$/50 μl, whereas the protein amplification-ELISA detected BHV-1 in semen infected with a 10^{-4} dilution of the virus and with a titer of 0.79 $TCID_{50}$/50 μl. Thus, even without considering the different amounts of samples used in the two methods, there is a direct observation of a 1,000-fold increase in sensitivity by protein amplification-ELISA over traditional PCR. Further optimization of the ELISA using labeled IgG MAbs will reduce the total time requirement for the entire assay.

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