

Evaluation of baculovirus-expressed bovine herpesvirus-1 (BHV-1) glycoproteins for detection and analysis of BHV-1-specific antibody responses

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Abstract

Baculovirus (*Autographa californica* nuclear polyhedrosis)-expressed bovine herpesvirus-1 (BHV-1) glycoproteins B (gB), gC, and gD were developed and characterized. The recombinant proteins retained their antigenic properties as determined by immunoblotting against monoclonal antibodies. The proteins were examined as antigens for detection of BHV-1 infection and for the analysis of antibody responses to the individual viral proteins. A total of 115 bovine serum samples were tested for their reactivity with individual recombinant proteins from baculovirus-infected *Spodoptera frugiperda* (SF-9) cell lysates by enzyme-linked immunosorbent assay (ELISA), western blotting, and dot blotting assays. These serum samples were previously tested for BHV-1-specific antibodies by virus neutralization (VN) at the veterinary diagnostic laboratory. All 90 serum samples tested positive with VN were positive by ELISA against gC and gD, separately. However, reactivities of sera against gB were generally low and inconsistent. On the other hand, out of 25 sera that were negative with VN, 22 sera were consistent and gave negative results against gC or gD by ELISA, whereas reactivities with gB were inconsistent. Similar results were obtained when the sera were tested by western blotting and dot blotting. The positive sera consistently reacted strongly against gC and to a lesser extent gD. These results suggest that baculovirus

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expressed gC from infected cell lysate can be used as a potential diagnostic antigen for detection of anti-gC-specific antibody responses in BHV-1 infected cattle. © 1998 Elsevier Science B.V.

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1. Introduction

Bovine herpesvirus-1 (BHV-1), also known as infectious bovine rhinotracheitis virus (IBRV), is associated with a wide variety of clinical syndromes including respiratory, genital, nervous, and multisystemic infections in cattle (Kahrs, 1977). Bovine herpesvirus-1 infection constitutes one of the most important diseases of cattle and causes significant economical losses to livestock worldwide. The virus envelope contains several glycoproteins, of which gB, gC, and gD have been identified as the major immunogens recognized by sera from infected cattle (Collins et al., 1985a; van Drunen Littel-van den Hurk and Babiuk, 1986; Marshal et al., 1986). Glycoproteins gB and gD are essential for virus replication and are responsible for inducing neutralizing antibody responses in the host. Although gC is dispensable for replication and induces lower neutralizing antibody response, it is immunodominant and is believed to play a role in initial attachment (reviewed in Tikoo et al., 1995). These properties make the three proteins excellent target antigens for detection and analysis of BHV-1 immune responses.

Diagnosis of BHV-1 infection has been based on virus isolation and serological tests including virus neutralization (VN), fluorescent antibody technique, and enzyme-linked immunosorbent assay (ELISA). Although VN is the most widely used test, it is expensive, time-consuming, and insensitive (Khars, 1982; Cho and Bohac, 1985; Collins et al., 1985b, c; Reigel et al., 1987). Several forms of ELISA have been described for the detection and diagnosis of BHV-1 infection in cattle (Bolton et al., 1981; Reigel et al., 1987; Collins et al., 1988; Ungar-Waron and Abraham, 1991). With the recent development of gene-deleted and recombinant live virus vaccines, the use of individual viral protein antigens will prove important for diagnostic evaluation of immune responses to help differentiate vaccinated from infected animals (Kit and Kit, 1991). In this study, we examined the utility of individual recombinant baculovirus (*Autographa californica* nuclear polyhedrosis)-expressed BHV-1 glycoproteins gB, gC, and gD as antigens for detection and analysis of specific antibody responses. Lysates from baculovirus-infected insect (*Spodoptera frugiperda*) cells expressing the proteins were assayed against field bovine sera by ELISA, dot blot, and western blot. Results of the assays obtained for 115 sera were correlated with results of VN test performed at the veterinary diagnostic laboratory at Auburn, Alabama. The findings have demonstrated differences in the properties of the three proteins as diagnostic antigens.

2. Materials and methods

2.1. Construction of transfer plasmids

Genes encoding the Cooper (Colorado-1) strain of BHV-1 gB, gC, and gD were cloned from *Hind*III genomic fragments provided by Dr. William Lawrence of the University of

Pennsylvania as described previously (Lawrence et al., 1986; Fitzpatrick et al., 1989; Tikoo et al., 1990; Abdelmagid et al., 1995). All cloning and DNA manipulation techniques were performed following standard procedures (Sambrook et al., 1989). The 3 Kb *NotI*–*AatII* DNA fragment containing gB, the 2.4 Kb *BamHI*–*EcoRI* DNA fragment containing gC, and the 1.3 Kb DNA fragment containing gD open reading frames (ORFs) were cloned in frame into pFastBac plasmids (Gibco BRL) under the polyhedrin gene promoter. In frame cloning and orientation were verified by asymmetric restriction site mapping and/or DNA sequencing of plasmid-insert junctions. Recombinant pFastBac plasmids served as transfer vectors to insert the glycoprotein genes into baculovirus DNA (Luckow, 1993).

2.2. Development of recombinant baculoviruses

Recombinant baculoviruses were generated using the Bac-to-Bac system developed by GibcoBRL following the manufacturer's recommendations (Luckow, 1993). Briefly, DNA from pFastBac plasmids were used to transform DH10Bac competent *E. coli* cells, already containing baculovirus composite (shuttle) plasmid vector bMON14272 called bacmid. By transposition, the glycoprotein genes were inserted into the bacmid plasmids to yield recombinant bacmids. DNA from recombinant bacmids was isolated and used for transfection of SF-9 insect cells. SF-9 cells were transfected with 1 µg of bacmid DNA using cationic liposomes (Lipofectin; GibcoBRL) according to the manufacturer's specifications. The transfection mixture was added to 1×10^6 SF-9 cells in a well of a 6-well tissue culture plate supplemented with SF900 II medium without serum and incubated for 5 h at 27°C. Baculovirus harvested from transfected cells was plaque titrated, analyzed for gene recombination by hybridization, and assayed for protein expression by immunoblotting as described previously (Abdelmagid et al., 1992, 1995).

2.3. Preparation of antigens

Antigens from recombinant proteins were prepared by infecting a suspension of SF-9 cells with 5 multiplicity of infection (m.o.i) of each recombinant baculovirus and cells were incubated in SF900 II medium by shaking at 27°C. Cells were harvested 72 h post-infection when viability reached 50% as determined by Trypan blue dye exclusion. The harvest time which gives the highest protein yield was predetermined by a time course of protein expression. Positive control antigens were prepared by infecting Madin Darby Bovine Kidney (MDBK) cells with 2 m.o.i of plaque purified BHV-1 (Cooper strain) and harvesting cells when CPE was complete. Infected SF-9 and MDBK cells were centrifuged at 1000 rpm for 10 min in a table top centrifuge and the pellets were suspended in TNE buffer (10 mM Tris–HCl, pH 7.8, 100 mM NaCl, 1 mM EDTA, 1% NP40) supplemented with protease inhibitors, phenylmethylsulfonyl fluoride (PMSF), Leupeptin, and Pepstatin A (Sigma) following supplier's recommended concentrations. The suspension was briefly sonicated and centrifuged at 14 000 rpm in a microcentrifuge for 30 min. The supernatant containing antigen was diluted in the proper buffer for ELISA, western blotting, or dot blotting as described below.

2.4. ELISA

Preliminary checker-board titration experiments were conducted to determine optimum volumes and reagent concentrations by using glycoprotein specific monoclonal antibodies (Mabs) and known control positive and negative bovine sera. The control sera were determined by pretesting against BHV-1-infected cell antigens by ELISA and by VN. The best results were obtained when approximately 300 ng of recombinant baculovirus-infected SF-9 cell or 100 ng of BHV-1 infected cell antigens were used to coat each well. Antigens were diluted in 0.05 M Sodium carbonate pH 9.6, a 100 μ l volume was added to each well and incubated for 1 h at 37°C and then was kept overnight at 4°C. Plates were blocked with 1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS) for 2 h. Test sera or Mabs were diluted in PBS-0.05% Tween 20 (PBST) and added to the plate. Sera were tested as a single 1:200 dilution or serial 10-fold dilutions. The 1:200 serum dilution was predetermined in pilot experiments to be the lowest bovine serum dilution which did not give false positive when control sera were serially diluted and tested against BHV-1-infected cell antigens. A prestandardized dilution of peroxidase-conjugated rabbit anti-bovine IgG, or goat anti-mouse IgG in PBST was added to the wells and the reaction was developed using *o*-phenylenediamine dihydrochloride (OPD) substrate in citrate buffer (Sigma) at 0.4 mg/ml. Optical densities at 405 nm wavelength were determined by using a microplate reader (Dynatech Laboratories, Alexandria, VA). Results obtained for bovine sera were compared with VN, western blotting, and dot blotting assays.

2.5. Western blotting

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed as previously described (Abdelmagid et al., 1995). Briefly, proteins extracted from baculovirus-infected-SF-9 cells and from BHV-1 infected MDBK cells were electrophoresed on a 12% gel, transferred to nitrocellulose (NC) membrane, and probed with monoclonal antibodies specific to BHV-1 gB, gC, or gD as controls or with test bovine sera. The Mabs were previously developed and characterized (van Drunen Littel-van den Hurk et al., 1984; Abdelmagid et al., 1992). When bovine sera were tested, the membranes were blocked with 1% gelatin, 2% horse serum (HS), 0.05% Tween 20 in TBS (TBST). Subsequent steps were performed with the biotin–avidin system using a kit from Vector Laboratories (Burlingame, CA).

2.6. Dot blotting

Antigens from SF-9 cells expressing gB, gC, and gD were prepared as described above and analyzed by dot blot microfiltration apparatus from Bio-Rad. Optimum dilutions in TBS of each antigen were predetermined by a checker-board dot blot titration. A 100 μ l volume of each antigen per well was loaded on NC membrane. The antigen was allowed to react for 45 min and removed by vacuum suction following the manufacturer's recommendations. The membrane was blocked and tested for reactivity against test bovine sera serially diluted in TBST. Immunostaining was carried out as described for western blotting.

3. Results

3.1. Development of baculoviruses and analysis of recombinant proteins

Transformation of DH10Bac *E. coli* with recombinant pFastBac plasmids resulted in the generation of recombinant shuttle vectors, bacmids, carrying BHV-1 gB, gC, and gD genes. Transfection of SF-9 cells with recombinant bacmid DNA has yielded recombinant baculoviruses carrying each of the three genes. Hybridization with ^{32}P -labelled specific DNA probes showed the recombinant baculoviruses to contain BHV-1 gB, gD, and gC genes, individually. The identity of gB, gC, or gD protein expressed by each baculovirus was analyzed by western blotting (Fig. 1) using glycoprotein specific monoclonal antibodies (anti-gB Mab mixture 83 and 94, anti-gC Mab mixture 10B, 24, and 38, and anti-gD Mab mixture R54 and 3D9S). Anti-gB Mabs reacted with three protein bands with apparent molecular weights of 35 Kd, 65 Kd, and 110 Kd which represent the three subunits that make BHV-1 gB. Anti-gC and anti-gD Mabs recognized a 78 Kd and a 75 Kd protein bands, respectively. The recombinant proteins appear to be reduced in molecular sizes when compared with the native viral proteins. However, their reactivities indicate that they carry antigenic properties similar to the native proteins. The anti-gB Mab reactivity against recombinant gB gave a slightly decreased signal when compared with gC and gD reactivities.

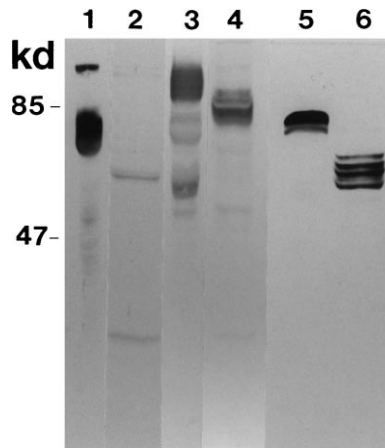


Fig. 1. Western blot analysis of baculovirus-expressed BHV-1 glycoproteins. Recombinant baculovirus-infected SF-9 cells expressing BHV-1 gB, gC, or gD and control BHV-1-infected MDBK cells were electrophoresed on 12% denaturing SDS-PAGE, transferred to nitrocellulose membranes, and probed with Mabs. Biotinylated secondary anti-mouse antibodies were added and the reactive protein bands were detected with peroxidase/4-chloro-1-naphthol enzyme–substrate system. Lanes 1 and 2 show reactivities of anti-gB Mab mixture 83 and 94 against BHV-1 and recombinant gB, respectively. Lanes 3 and 4 show reactivities of anti-gC Mab 24 and 38 mixture against BHV-1 and recombinant gC, respectively. Lanes 5 and 6 show reactivities of anti-gD Mab R54 and 3D9S against BHV-1 and recombinant gD, respectively.

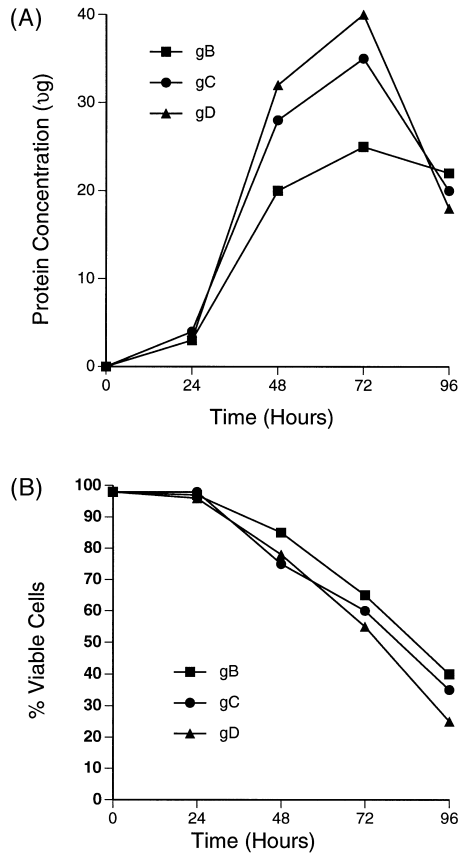


Fig. 2. Expression of BHV-1 gB, gC, and gD and percent viability of SF-9 cells after infection with baculovirus recombinants. Protein concentrations were estimated by densitometry against protein standards and plotted as amount of protein per 5×10^6 infected SF-9 cells as a function of time (A). The viability of SF-9 cells over the time course of infection with baculovirus recombinants expressing gB, gC, and gD is shown (B). Cell samples were removed at the time indicated and stained with Trypan blue. Cell viability was estimated as the number of cells that excluded the dye versus the total number of cells per field.

3.2. Protein expression and preparation of antigens

Time course studies were performed to ascertain the optimal time for obtaining each recombinant protein. Suspension of SF-9 cells infected with five m.o.i of each baculovirus was sampled every 24 h post infection for 4 days. At each time point cells were counted and viability was determined by trypan blue exclusion staining. The cells were pelleted and their recombinant protein content was determined by western blotting and densitometric scanning of reactive bands and comparison with protein of known concentration. Total infected cell protein concentration was determined by a kit from Bio-Rad. Similar levels of protein expression were observed for gC and gD (Fig. 2). Approximately 35–40 µg of reactive proteins were obtained from 5×10^6 infected SF-9

cells. The relative recombinant protein concentration from the total infected cell proteins was estimated to be approximately 15% for gC or gD and 11% for gB. The level of gB protein expression was comparatively low, although cell viability curve showed infection pattern similar to gC and gD.

3.3. ELISA

One hundred and fifteen bovine serum samples previously tested for BHV-1 antibodies by VN at the veterinary diagnostic laboratory (Auburn, AL) were retested by ELISA against recombinant baculovirus-expressed gB, gC, and gD (Table 1). ELISA results were categorized as high positive, low positive, and negative based on the absorbance values obtained for control bovine sera against BHV-1-infected MDBK cell antigens. Absorbance values obtained for control negative sera were considered as negative values for test serum samples. The high and low positive absorbance values were determined according to the reactivity of control positive sera with a range of VN titers. Results of a single serum dilution (1:200) showed that the 90 sera which tested positive by VN were positive by ELISA against gC and gD giving average absorbance (at 405 nm) values of 0.95 and 0.82, respectively. Reactivities with gB were weak, 0.65 in average, and inconsistent with VN results (Table 1). Absorbance values with control-uninfected SF-9 cell proteins were at ≤ 0.20 and consistent. Positive control antigen made of BHV-1-infected MDBK cell gave the highest average absorbance of 1.2. Reactivity of all 90 sera with uninfected MDBK cells were less than 0.10. ELISA titers obtained against gC and gD were consistent with the VN titers and ranging between 5×10^3 and 5×10^5 . Out of 25 sera tested negative by VN, 22 samples gave absorbance of ≤ 0.20 against gC and gD and considered negative. However, 3 samples showed low positive level absorbance between 0.5 and 0.6. Similar results were obtained against control BHV-1 antigen. Some negative sera showed nonspecific reactivities against gB (Table 1).

Table 1
Summary of comparative mean reactivities of bovine sera against individual baculovirus-expressed BHV-1 glycoproteins by ELISA

Serum samples tested		
Antigen	90 sera (positive by VN) Mean absorbance value \pm SD	25 sera (negative by VN) Mean absorbance value \pm SD
BHV-1	1.20 \pm 0.15	≤ 0.10 (22), 0.62 \pm 0.14 (3 [*])
gB	0.65 \pm 0.35	≤ 0.20 (15), 0.44 \pm 0.30 (10)
gC	0.95 \pm 0.19	≤ 0.20 (22), 0.54 \pm 0.15 (3 [*])
gD	0.82 \pm 0.20	≤ 0.20 (22), 0.50 \pm 0.17 (3 [*])

A total of 115 serum samples previously tested by virus neutralization for BHV-1 antibodies were retested by ELISA against antigens from BHV-1-infected MDBK cells and recombinant baculovirus-infected SF-9 cells expressing BHV-1 gB, gC, and gD. ELISA absorbance values were obtained at 405 nm wave length. All sera tested positive by VN had absorbance values of ≤ 0.20 against control uninfected SF-9 cells and ≤ 0.10 against control uninfected MDBK cells. The asterisk (*) denotes the same serum samples. Data were expressed as least square means \pm standard errors. Significant difference was determined at $p \leq 0.01$.

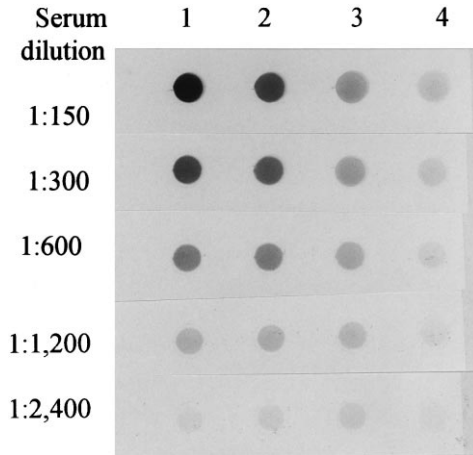


Fig. 3. Reactivity of bovine serum with recombinant proteins by western blotting. The figure depicts the reactivity of a representative positive bovine serum sample diluted 1:200 in TBST and tested against recombinant baculovirus-infected SF-9 cells expressing BHV-1 gB (lane 2), gC (lane 3), and gD (lane 4). Reactivity against control BHV-1-infected MDBK cell lysate is shown in lane 1. Western blotting was carried out as described for Fig. 1.

3.4. Western blotting

Twenty-five sera representing positive samples with different VN titers and negative samples were tested by western blotting. All positive sera diluted 1:200 reacted relatively strongly against gC when compared to reactivity with gB and gD (Fig. 3). No detectable reactivity was observed against uninfected SF-9 cell proteins. Fig. 3 shows the immunoblotting results of a representative positive sample against the three antigens. BHV-1 infected MDBK is included as positive control.

3.5. Dot blotting

Twenty-five sera representing positive samples with different VN titers and negative samples were tested against recombinant gB, gC, and gD by dot blotting. The results obtained are represented in Fig. 4. All positive sera consistently reacted strongly with gC, having higher titers when compared to gD. Titers obtained against gC were comparable to those obtained against BHV-1 infected cell proteins. Reactivity with gB was weak and inconsistent with VN results. No detectable reaction of negative sera is observed against uninfected SF-9 and uninfected MDBK cells. Results of serum titration by dot blot are consistent with ELISA and western blotting.

4. Discussion

Previous studies have demonstrated BHV-1 glycoprotein gB, gC, and gD as the major targets of the bovine humoral immune responses (Collins et al., 1985a; van Drunen Littel-

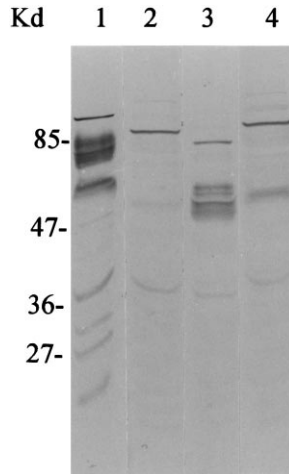


Fig. 4. Reactivity of bovine sera with recombinant proteins by dot blot. A predetermined concentration of antigen from recombinant baculovirus-infected SF-9 cells expressing BHV-1 gC (lane 2), gD (lane 3), and gB (lane 4) were added per well on NC membrane using dot blot apparatus. The NC membrane was blocked and tested against a selected representative positive bovine serum sample serially diluted in TBST as indicated in the figure. Reactivity of the serum against control BHV-1 infected MDBK cell lysate is shown in lane 1. Immunostaining was carried out as described for western blotting in Fig. 1.

van den Hurk and Babiuk, 1986; Marshal et al., 1986). All three glycoproteins stimulated the production of varying levels of specific antibody responses in mice, rabbits, and cattle (reviewed in Tikoo et al., 1995). In the present study, we analyzed the antigen specificities of cattle immune responses to BHV-1 by testing a 115 field bovine sera against baculovirus-expressed recombinant gB, gC, and gD proteins. The purpose of these experiments was to examine the utility of the individual recombinant proteins as diagnostic antigens for detection of specific BHV-1 antibodies. The use of individual proteins as antigen for diagnosis is becoming especially important with the advent of live gene-deleted virus vaccines (Kit and Kit, 1991). The baculovirus-expressed proteins retained their native antigenic properties when tested against known Mabs by immunoblotting (Fig. 1). Recombinant gB showed a relatively weak signal against Mabs when compared with gC and gD. The highest level of expression of each protein was obtained when SF-9 cells were infected and harvested at 72–96 h post infection (Fig. 2).

Bovine sera previously tested for BHV-1 antibodies by VN at the diagnostic laboratory were tested against baculovirus-expressed proteins by plate ELISA, dot blotting, and western blotting. The antigen used in all tests was prepared from recombinant baculovirus-infected cell lysates rather than purified proteins to test its suitability for detection of BHV-1 specific antibodies. If results are successful, the use of antigen will be feasible due to reduction in time and cost needed to purify the proteins. Our results have shown that all sera which tested positive by VN consistently tested positive with gC and gD by indirect ELISA, dot blotting, and western blotting. Reactivity with gC antigens was strongest and most consistent when compared to gD. Average absorbance values of

positive sera against gC were higher than those against gD and slightly less than control reactivity against BHV-1-infected MDBK cell antigens (Table 1). ELISA titers against gC ranged between 5×10^3 and 5×10^5 generally a half to one log less than the titers against control BHV-1 antigens but consistent with VN titers. Results with gC are compatible with previous findings that gC is one of the immunodominant virus glycoproteins during natural infection (Collins et al., 1985a; van Drunen Littel-van den Hurk and Babiuk, 1986). In contrast, absorbance values against gB were low and less consistent by all immunoassays. These results are not in agreement with the fact that gB induces a consistent and relatively high neutralizing antibody response in BHV-1 infected cattle (Collins et al., 1985a; van Drunen Littel-van den Hurk and Babiuk, 1986). The poor reactivity of recombinant gB may be due in part to the relatively low concentration of gB in infected cell preparation when compared to gC and gD. It is also likely that baculovirus-expressed gB may have lost some of its native conformation which resulted in partial loss of antigenicity. This is evident by the weak signal against Mabs in immunoblotting (Fig. 1).

Absorbance values against control uninfected SF-9 cell lysates were higher (≤ 0.20) than those against uninfected MDBK cells (≤ 0.10) (Table 1). However, this level of reactivity was always consistent. Three out of 22 bovine sera which were negative by VN, gave low positive (0.50) absorbance when tested by ELISA against BHV-1, gC, and gD. Similar results were obtained when sera were tested by dot blot. This may be due to that ELISA and dot blot are more sensitive assays than VN in detecting antibodies.

5. Conclusions

In conclusion, baculovirus-expressed BHV-1 gC and to a lesser extent gD are good diagnostic antigens for detection of BHV-1-specific antibodies. The use of infected insect cell lysate as antigens in ELISA and dot blot yielded specific results which are comparable to VN tests. On the other hand, results with baculovirus-expressed gB were weak and inconsistent. Different expression strategies need to be explored to increase the level of gB expression and its antigenicity. Reactivity of sera with uninfected insect cells showed relatively low background. These results indicate that individual baculovirus-expressed recombinant proteins can be used to evaluate immune responses of cattle against specific viral envelope glycoproteins. This property of diagnostic antigens is especially important with the use of gene-deleted virus vaccines to differentiate vaccinated from infected animals.

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