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Antigen Capture Competitive Enzyme-Linked Immunosorbent Assays Using Baculovirus-Expressed Antigens for Diagnosis of Bluetongue Virus and Epizootic Hemorrhagic Disease Virus

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Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are orbiviruses that infect both livestock and wild ruminants. Antigenic cross-reactivity between BTV and EHDV often results in serologic misdiagnosis. Competitive enzyme-linked immunosorbent assays (c-ELISAs) show increased sensitivity and specificity for the identification of these viral diseases; however, the preparation of cell culture-derived viral antigen for these tests is laborious and variable from batch to batch, and the resulting antigen may be infectious. To overcome these problems, the genes coding for a structural protein, VP7, of BTV and EHDV were cloned into baculovirus and the recombinant proteins were expressed in Sf9 cultured insect cells. Recombinant viral proteins released into the baculovirus-infected Sf9 cell culture supernatant were used in antigen capture c-ELISAs (Ag Cap c-ELISA) tests that specifically detected antibody in the serum of cattle experimentally infected with BTV and EHDV. The diagnostic utility of the Ag Cap c-ELISA was demonstrated by comparison with a commercial c-ELISA. The Ag Cap c-ELISA offers the advantages of using an easily produced, easily standardized, noninfectious antigen that does not require further purification or concentration.

Bluetongue virus (BTV) and epizootic hemorrhagic disease virus (EHDV) are arthropod-borne orbiviruses that infect both domestic and wild ruminants (4, 9). Bluetongue is classified as a list A disease by the Office des Epizooties and can cause considerable economic consequences because of both the disease itself and the resulting restrictions in international livestock trade. Five serotypes of BTV (serotypes 2, 10, 11, 13, and 17) have been identified in the United States, while two serotypes of EHDV, designated EHDV-1 (New Jersey strain) and EHDV-2 (Alberta strain), are known to be enzootic in the United States. Diagnosis of infections caused by these two viruses is often confounded because of their antigenic similarity (15). To overcome this problem, competitive enzyme-linked immunosorbent assay (c-ELISA) procedures have been developed for the serologic diagnosis of infections caused by these two groups of viruses (1, 2, 12, 18). Because of their sensitivity and specificity, c-ELISAs have become the assays of choice for serologic monitoring of infections caused by these viruses (3, 16). ELISAs depend on the incorporation of a suitable antigen into the assays. Most of the c-ELISAs for BTV and EHDV use monoclonal antibody (MAb) to VP7, which is highly conserved among members of these two serogroups. Typically, antigens for BTV and EHDV ELISAs are produced by infection of susceptible cultured cells followed by extraction and purification of virus or viral antigen. This process is time-consuming and requires large volumes of cells and reagents, and the antigens can vary in quality and quantity from preparation to preparation. In addition, such antigen preparations may still be infectious and must be handled accordingly. The production of suitable viral antigen in heterologous expression systems is an attractive alternative that may overcome these problems. Vaccinia virus and baculovirus were shown to express BTV and EHDV proteins that could be used as antigens to bind antibody in an indirect ELISA format (5, 13, 14). The VP7 protein from BTV expressed in yeast was shown to be a suitable antigen in more specific blocking ELISAs and c-ELISAs for serum antibody detection (1, 8).

Baculoviruses are widely used vectors because recombinant proteins are expressed in large amounts in infected insect cells. We cloned the genes that code for VP7 of both BTV and EHDV into baculovirus vectors and were interested in determining if the expressed VP7 proteins would be suitable as antigens in a c-ELISA format for detection of serum antibody to BTV and EHDV. We report the use of recombinant VP7 proteins, released into the cell culture supernatant of baculovirus-infected Sf9 cells, in antigen capture (Ag Cap) c-ELISAs that detect serum antibody to either BTV (BTV Ag Cap c-ELISA) or EHDV (EHDV Ag Cap c-ELISA). The Ag Cap c-ELISAs offer the advantages of using easily produced, high-titer, noninfectious antigen directly from baculovirus-infected Sf9 cell culture supernatant without further purification or concentration.

MATERIALS AND METHODS

Cloning of BTV and EHDV VP7 into a baculovirus expression vector. The genes coding for VP7 of BTV-11 and EHDV2 were reverse transcribed and PCR amplified. The amplification primers incorporated *Bam*HI and *Xho*I restriction sites into the 5′ and 3′ ends, respectively, that allowed baculovirus double restriction digests and directional ligations of the cDNA into the plasmid transfer vector, pBlueBacHis2B vector (Invitrogen). *Escherichia coli* was transformed with the vector containing the insert by electroporation and ampicillin and 5-bromo-4-chloro-3-indolyl-β-D-galactopyronoside (X-Gal) selection. The presence and orientation of inserts in selected clones was determined by a PCR colony screening method and by sequence analysis. Positive clones were propagated, and the plasmids were purified using a commercial procedure (Qiagen). Sf9 cells were transfected with the purified plasmids, baculovirus from the trans-

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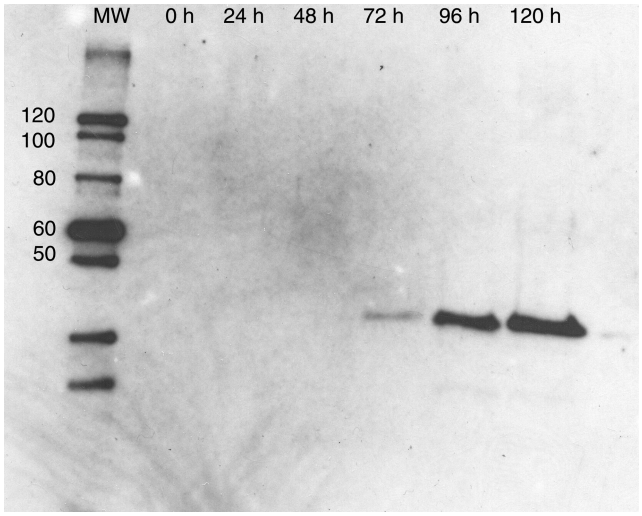

FIG. 1. Western blot detection of baculovirus-expressed EHDV VP7. Sf cells were infected with the recombinant baculovirus, and samples of cell culture supernatant fluid were analyzed, at the indicated time after infection, by Western blotting for the presence of expressed EHDV VP7 with a mouse MAb. MW, molecular weight in thousands.

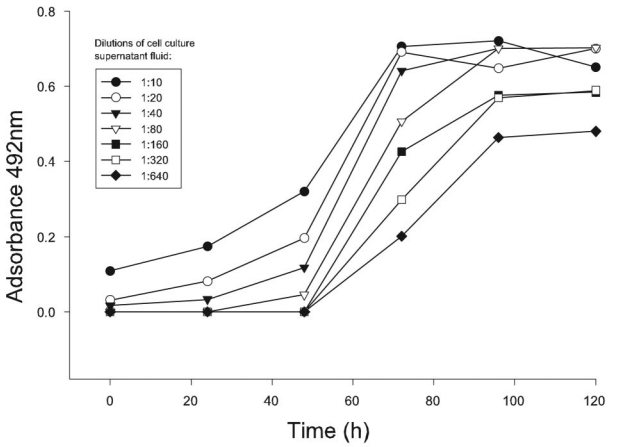

FIG. 3. Ag Cap ELISA detection of EHDV VP7 in Sf9 culture supernatant fluids. Sf9 cells were infected with recombinant baculovirus, samples of cell culture supernatant fluid were collected at the indicated times postinfection, and dilutions (1:10 to 1:640) of this material were analyzed for the presence of expressed EHDV VP7 by Ag Cap ELISA.

fected cells was harvested, and recombinant baculovirus was plaque purified and propagated. The presence of recombinant virus was verified by PCR. Stock recombinant virus was prepared by infection of Sf9 cells, and the titer was determined by a plaque assay. Protein expression was accomplished by infecting Sf9 cells with a multiplicity of infection of 0.5. Samples of supernatant fluid were removed every 24 h for 5 days, and protein expression was determined by Western blotting and Ag Cap ELISA.

Western blotting. Samples from baculovirus-infected Sf9 cell cultures were electrophoresed using the discontinuous sodium dodecyl sulfate-polyacrylamide gel electrophoresis system of Laemmli (7) and transferred by electroblotting to nitrocellulose membranes. Recombinant viral proteins were detected by chemiluminescence with the West Femto kit (Pierce), using either a rabbit polyclonal antibody to BTV-11 or a mouse MAb (4F4.H1) (12) to EHDV-2, VP7.

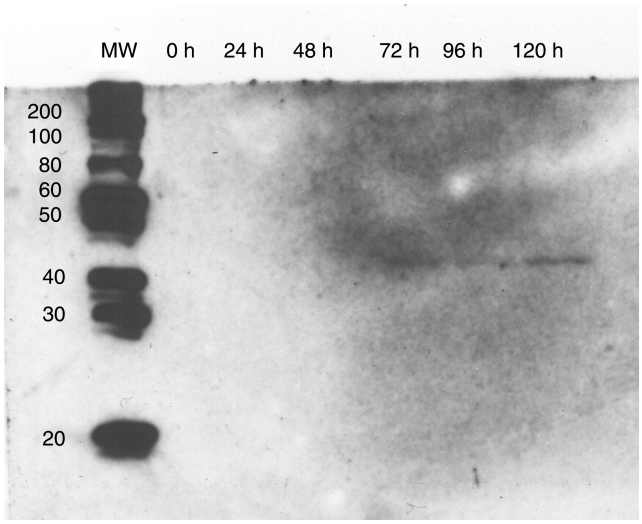

FIG. 2. Western blot detection of baculovirus-expressed BTV VP7. Sf9 cells were infected with the recombinant baculovirus, and samples of cell culture supernatant fluid were analyzed, at the indicated time after infection, by Western blotting for the presence of expressed BTV VP7 with a rabbit polyclonal antibody. MW, molecular weight in thousands.

ELISA procedures. An Ag Cap ELISA format (10, 11) was used to detect recombinant VP7 expressed in the Sf9 cell culture supernatant fluid samples removed at various times after infection. Briefly, the wells of microtiter plates were coated with polyclonal rabbit anti-BTV or anti-EHDV serum and VP7 antigen was captured from dilutions of the baculovirus infected Sf9 cell culture supernatant fluid samples. The relative amounts of VP7 antigen in these fluids were determined by measuring the optical density following sequential additions of mouse MAbs specific for VP7 of BTV or EHDV, biotinylated goat anti-mouse, peroxidase-conjugated streptavidin, and *ortho*-phenylenediamine (OPD).

The Ag Cap c-ELISAs were done as previously described for the c-ELISA (12) with the following modification. Instead of being adsorbed directly to the wells of 96-well Immulon II microtiter plates, the antigen was captured from baculovirus-infected Sf9 cell culture supernatant fluids with rabbit polyclonal antibody (anti-BTV or anti-EHDV) that had been adsorbed to the wells of the plate. The remaining steps of the test were carried out as previously described. Briefly, MAb and test serum samples were allowed to compete for binding to the captured antigen. This was followed by reaction with biotinylated goat anti-mouse antibody and peroxidase-conjugated streptavidin. Optical density (OD) measurement of the color change following addition of OPD allowed calculation of the percent inhibition (PI) of binding of the MAb to the captured antigen and a relative measure of the concentration of specific antibody in the test serum sample. Percent inhibition was calculated as $PI = 100 - (OD_{492}$ of test serum/OD_{492} of negative serum$) \times 100$ (12). Optimal reagent concentrations for the Ag Cap c-ELISA were determined by cris-cross serial dilutions (6). It was determined that a 1:200 dilution of rabbit anti-BTV or rabbit anti-EHDV was optimal for coating the wells of the microtiter plates. A 1:50 dilution of culture supernatant from Sf9 cells infected for 5 days with recombinant baculovirus was used as a source of antigen. Dilutions of 1:50 for mouse MAbs and 1:5 to 1:10 for test serum samples were determined to be optimal.

Antibodies. Six steers were experimentally infected with BTV-10, BTV-11, BTV-13, BTV-17, EHDV-1, or EHDV-2, and serum samples were collected weekly for a total of 7 weeks to obtain samples for developing the Ag Cap c-ELISAs. Antibody responses in these experimentally infected animals were verified by measuring the plaque neutralization titers. A mouse MAb designated 1AA4.E4, specific for VP7 of BTV, was used in the BTV Ag Cap c-ELISA, and a mouse MAb designated 4F4.H1, specific for VP7 of EHDV, was used in the EHDV Ag Cap c-ELISA (11, 12).

Diagnostic evaluation. The diagnostic utility of the Ag Cap c-ELISA was evaluated in a side-by-side comparison with a commercial c-ELISA (VMRD, Inc., Pullman, Wash.) for detection of serum antibody to BTV. Serum samples from sheep, cattle, and deer were tested in duplicate and scored as positive or

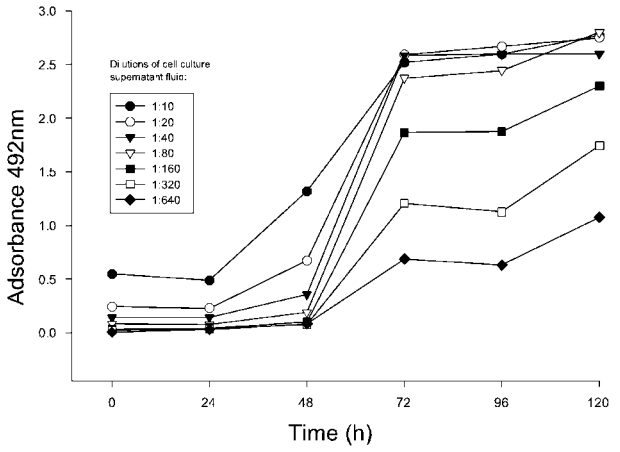

FIG. 4. Ag Cap ELISA detection of BTV VP7 in Sf9 culture supernatant fluids. Sf9 cells were infected with the recombinant baculovirus, samples of cell culture supernatant fluid were collected at the indicated times postinfection, and dilutions (1:10 to 1:640) of this material were analyzed for the presence of expressed BTV VP7 by Ag Cap ELISA.

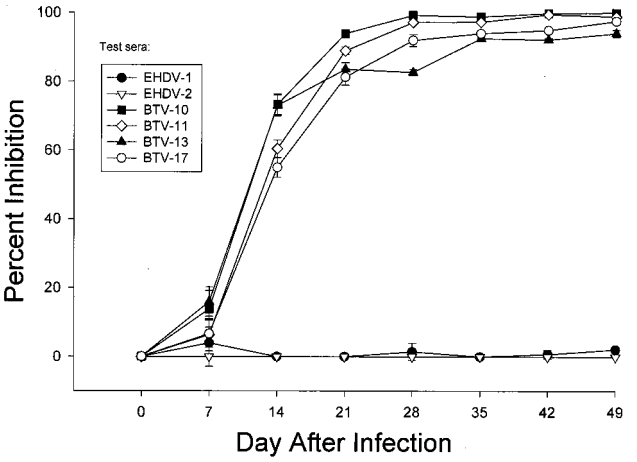

FIG. 6. Ag Cap c-ELISA for detection of antibodies to BTV. Baculovirus-expressed BTV VP7 was used in an Ag Cap c-ELISA to detect antibodies in the serum of cattle that had been infected with BTV and EHDV. The error bars show standard deviations from three assay replications for each serum sample at each time point.

negative by using 50 PI as a cutoff, as specified in the instructions for the commercial kit.

RESULTS

Expression of VP7. Infection of Sf9 cells with the recombinant baculoviruses resulted in the release into the cell culture supernatant of a protein of about the same size as VP7 of BTV or EHDV that could be detected by Coomassie blue staining of sodium dodecyl sulfate-polyacrylamide gels beginning 72 h postinfection (data not shown). Verification that this protein was VP7 was done by Western blotting and detection using antibodies specific for BTV or EHDV. The baculovirus-expressed EHDV-2 VP7 was first detected in supernatant fluid at 72 h after infection of the Sf9 cells. The amount of expressed VP7 detected in the supernatant fluid increased out to 120 h after infection, when the experiment was stopped (Fig. 1). A similar course was observed for the accumulation of baculovirus-expressed BTV-11 VP7 in the supernatant fluid of infected Sf9 cells, although the bands were very faint at both 72 and 96 h after infection (Fig. 2). A MAb was used for detection of the EHDV-expressed VP7, whereas a rabbit polyclonal antibody was used for detection of the BTV-expressed VP7, since the available MAb to BTV did not react in the Western blot. The intensity of staining of expressed BTV VP7 was probably related to the reactivity of the rabbit polyclonal antibody. The Westerm blot results verified that recombinant VP7 was released into the infected cell culture supernatant. All supernatant fluids were harvested at 120 h after infection, since the Sf9 cell cultures showed significant cytopathologic effects at that time.

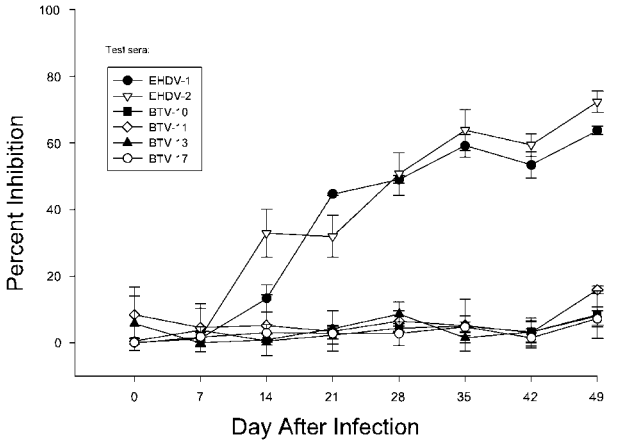

FIG. 5. Ag Cap c-ELISA for detection of antibodies to EHDV. Baculovirus-expressed EHDV VP7 was used in an Ag Cap c-ELISA to detect antibodies in the serum of cattle that had been infected with EHDV or BTV. The error bars show standard deviations from three assay replications for each serum sample at each time point.

The above results were confirmed by measuring the relative amounts of expressed VP7 in baculovirus-infected Sf9 supernatant fluids by an Ag Cap ELISA. Twofold dilutions (1:10 through 1:640) for each time point (0, 24, 48, 72, 96, and 120 h after infection) were tested for the relative amounts of baculovirus-expressed antigen released into the supernatant fluids. The release of VP7 into the cell culture supernatant fluids reached a plateau between 72 and 96 h after infection. In the plateau region (72 to 120 h after infection), the cell culture supernatant fluids could be diluted 1:80 without any significant loss of assay signal (Fig. 3 and 4).

Incorporation of the expressed VP7 into an Ag Cap c-ELISA. Initially, expressed VP7 was evaluated in a standard c-ELISA format by directly adsorbing the antigen from infected Sf9 supernatant fluid to the wells of 96-well plates. However, this did not result in adequate signal in the test. Use of more highly purified and concentrated antigen from either cell culture supernatant fluids or the infected Sf9 cells did not

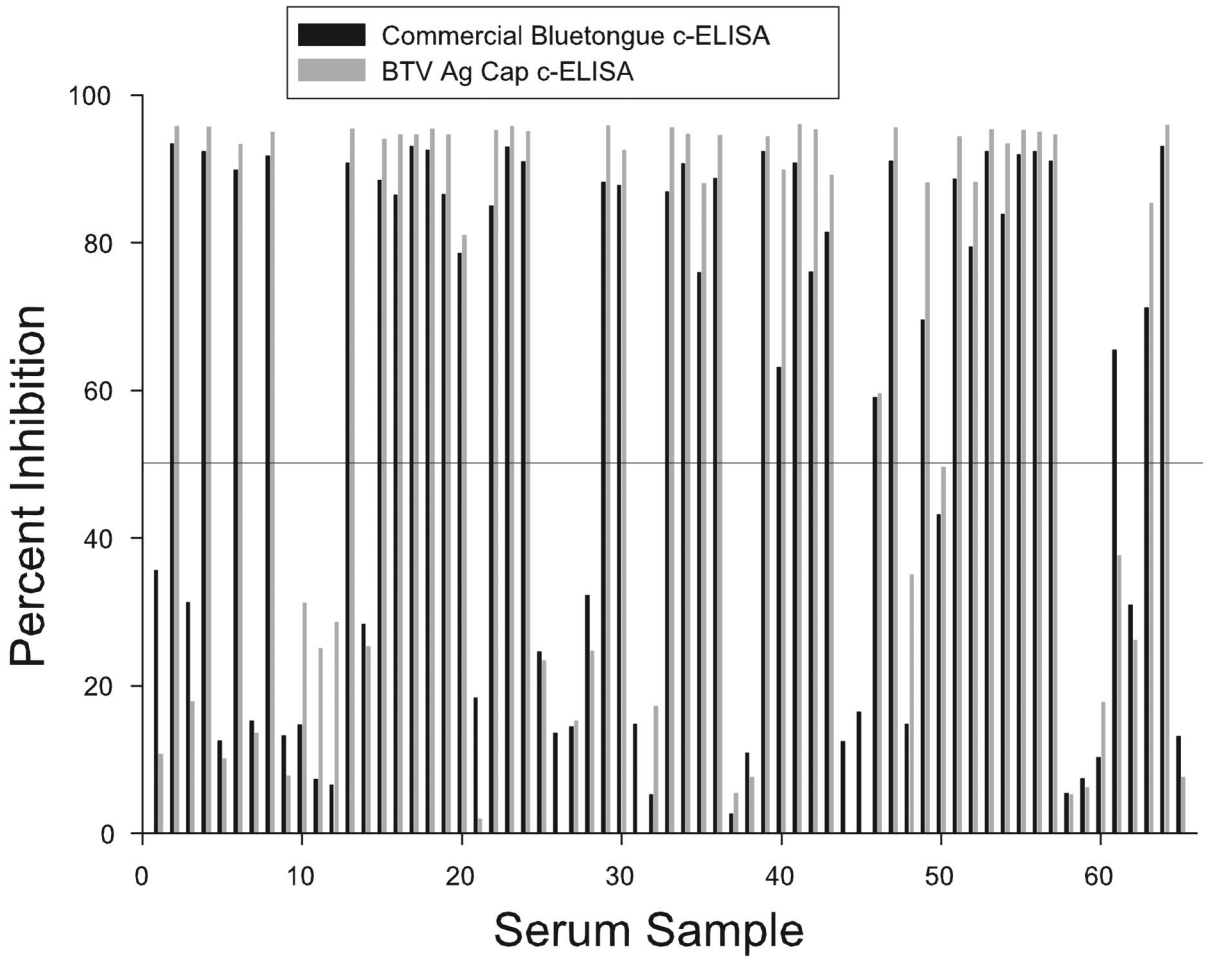

FIG. 7. Comparison of the BTV Ag Cap c-ELISA and a commercial bluetongue c-ELISA for detection of antibody in serum samples from cattle (samples 1 to 31), sheep (samples 32 to 45), and deer (samples 46 to 65). The line bisecting the graph indicates 50 PI and was used as the cutoff point for scoring samples as positive or negative.

significantly improve the signal (data not shown). Therefore, an initial Ag Cap step, in which baculovirus-expressed proteins in the culture supernatant fluid were trapped using rabbit polyclonal antibodies to either BTV or EHDV coated to the microtiter plate wells, was incorporated into the test. Following this step, the standard competition and detection steps of the c-ELISA were carried out. Incorporating the Ag Cap step into the c-ELISA allowed the detection of antibodies in the serum of individual cattle that had been experimentally infected with one of four serotypes of BTV or one of two serotypes of EHDV. The Ag Cap c-ELISA that incorporated the baculovirus-expressed EHDV VP7 was specific for the detection of EHDV serum antibodies, and the Ag Cap c-ELISA that incorporated the baculovirus-expressed BTV VP7 was specific for the detection of BTV serum antibodies. A specific antibody response could be detected by both assays as early as 14 days after infection. Homologous competition approached 100 PI between 21 and 28 days after infection in the BTV Ag Cap c-ELISA, while in the EHDV Ag Cap c-ELISA, it was approximately 60 to 70 PI between 35 and 49 days after infection. Heterologous cross-reactivity in the BTV Ag Cap c-ELISA was not significantly greater than 0 PI; in the EHDV Ag Cap c-ELISA it did not exceed 10 to 15 PI (Fig. 5 and 6). The specificity of the Ag Cap c-ELISAs was confirmed by detection of antibody in the serum of the experimentally infected animals by plaque reduction neutralization assays (data not shown).

Diagnostic utility of the Ag Cap c-ELISA. The diagnostic utility of the Ag Cap c-ELISA was evaluated by comparison with a commercially available c-ELISA (VMRD, Inc.) for the detection of antibody to BTV in 65 serum samples from cattle, sheep, and deer. There was excellent correlation between the BTV Ag Cap c-ELISA and the commercial c-ELISA. There was agreement in scoring all the samples as positive or negative (based on a 50 PI cutoff), except for one sample. Sample 61 from a deer was negative by the BTV Ag Cap c-ELISA but positive by the commercial bluetongue c-ELISA (Fig. 7). This sample was also negative for antibody to BTV but positive for antibody to EHDV by plaque reduction neutralization assays. In addition, it was positive for antibody to EHDV by the EHDV Ag Cap c-ELISA (data not shown).

DISCUSSION

The genes coding for the BTV and EHDV VP7 structural proteins were cloned into a baculovirus expression vector. Infection of Sf9 insect cells with these recombinant viruses resulted in the accumulation of the VP7 of BTV or EHDV in the cell culture supernatant fluids. These recombinant VP7 proteins were shown to be antigenically intact by Western blot analysis and were successfully used in Ag Cap ELISAs for the detection of serum antibodies to BTV and EHDV in cattle experimentally infected with these viruses.

Baculoviruses have been used to express a number of orbivirus proteins for structural and functional studies; however, their evaluation as potential diagnostic reagents has been limited. Baculovirus-expressed BTV VP7 was shown to bind guinea pig antibodies and baculovirus-expressed EHDV VP7 was shown to bind rabbit and bovine antibodies in indirect ELISAs (13, 14). MAbs produced against baculovirus-expressed recombinant EHDV VP7 were used in a standard c-ELISA with tissue culture-derived antigen to detect serum antibody in cattle experimentally infected with EHDV (20). To our knowledge, this is the first report of the use of baculovirus-expressed VP7 as the antigen in c-ELISAs for the detection of serum antibodies to BTV or EHDV.

Yeast-expressed BTV VP7 was shown to be a suitable antigen in a standard c-ELISA; however, antigen preparation required lysis of the yeast cells and partial purification of the antigen (1). The Ag Cap c-ELISAs reported here incorporate an Ag Cap step in a typical c-ELISA format. The addition of this step allowed the use of baculovirus-infected Sf9 culture supernatant fluid directly without additional purification or concentration. Attempts to improve the test by first purifying and concentrating VP7 from either the supernatant fluids or infected cells did not do so; however, these steps did add significant time and labor to the procedure. Purified and concentrated VP7 also did not increase the signal in a standard c-ELISA format without the Ag Cap step. Binding the expressed antigen directly to the wells of the microtiter plate may result in partial denaturation of the epitopes and inhibit binding of the detection MAb; however, capturing the VP7 with a bound polyclonal antibody may help preserve the antigenic integrity of the epitope. Lysates of BTV- and EHDV-infected cell cultures have also been used as sources of antigen in the Ag Cap c-ELISAs without further purification or concentration (data not shown).

The sensitivity and specificity of the BTV and EHDV Ag Cap c-ELISAs differed slightly. Some heterologous cross-reactivity was noted in the EHDV Ag Cap c-ELISA. Similar heterologous cross-reactivity has been detected in standard EHDV c-ELISAs (12, 17, 19). Because the maximum heterologous inhibition in the EHDV Ag Cap c-ELISA was not significantly greater than 15 PI, a cutoff threshold of 30 PI or greater should eliminate false-positive results in diagnostic applications. Homologous competition was better in the BTV Ag Cap c-ELISA than in the EHDV Ag Cap c-ELISA. Attempts to increase the sensitivity of the EHDV Ag Cap c-ELISA by further dilution of the MAb enhanced the sensitivity but also decreased the specificity (increased heterologous cross-reactivity). In addition, use of another MAb, recognizing a different epitope on VP7 of EHDV, resulted in slightly improved sensitivity but a loss in specificity (data not shown). These differences most probably reflect differences in the immune response of animals following infection by the two viruses.

The BTV Ag Cap c-ELISA performed comparably to a commercial bluetongue c-ELISA in side-by-side testing (98.5% agreement in scoring positives and negatives). The one discordant result may have been due to cross-reactive antibody to EHDV that produced a false-positive result.

The advantages of the Ag Cap c-ELISAs over standard c-ELISAs relate to the antigen. Several liters of cell culture supernatant from recombinant baculovirus-infected Sf9 cells can be easily produced in a few days. Once the supernatant is harvested and the titer of antigen is determined, this material can be used directly, without further purification, as a source of noninfectious antigen in the Ag Cap c-ELISA. The Ag Cap step should allow the use of other crude antigen preparations (infected mammalian cell culture lysates) in the test. The Ag Cap c-ELISA protocol should also be adaptable to diagnostic assays for other viruses.

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