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ENZYME-LINKED IMMUNOSORBENT ASSAY FOR EFFICIENT DETECTION OF ANTIBODY TO BLUETONGUE VIRUS IN PRONGHORN (*ANTILOCAPRA AMERICANA*)

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ABSTRACT: An indirect enzyme-linked immunosorbent assay (ELISA), using cell-associated viral antigen, was developed for detection of antibody to bluetongue virus (BTV) in field-collected pronghorn (*Antilocapra americana*) sera. To test the applicability of the ELISA to seroepizootiologic studies, pronghorn serum samples from three Wyoming counties (USA) were tested. Bluetongue virus ELISA results were compared to those of the bluetongue immunodiffusion assay. Discrepant serum samples were retested for reaction to either BTV or epizootic hemorrhagic disease virus. The pronghorn BTV ELISA gave rapid, quantitative, objective results and should facilitate testing large numbers of sera for BT diagnostic and seroepizootiologic studies.

Key words: Pronghorn, *Antilocapra americana*, bluetongue, seroepizootiology, indirect enzyme-linked immunosorbent assay (ELISA), diagnostic test, field study.

INTRODUCTION

Bluetongue (BT) is an arthropod-borne orbiviral disease of domestic and wild ruminants. Pronghorn antelope (*Antilocapra americana*) are commonly affected by bluetongue virus (BTV) (Thorne et al., 1988) and are an important source of information in studying BT antibody prevalence where pronghorn grazing areas overlap those of domestic ruminant livestock. It has been shown that the geographical distribution of BT in wild ruminants generally parallels that of the disease in livestock (Trainer and Jochim, 1969).

Diagnosis of BTV infection relies primarily on detection of group-specific anti-BTV antibodies (Anderson, 1985; Della-Porta et al., 1985; Jochim, 1985). Bluetongue immunodiffusion (BTID) (Pearson and Jochim, 1979), is the official USDA assay for testing sera for import/export and is often used in epizootiologic studies. False negative results, due to the assay's limited sensitivity (Afshar et al., 1987; Poli et al., 1982), and false positive results, thought to be due to cross-reactivity of the antigenically similar epizootic hemorrhagic disease virus (EHDV) (Afshar et al., 1987; White et al., 1985), can make the BTID unreliable. Described herein is an indirect enzyme-linked immunosorbent assay (ELISA), using cell-associated (CA) viral antigen for the detection of antibodies to BTV in field-collected pronghorn sera. The samples were also tested against uninfected CA antigen to determine if background reactions caused false positive results.

MATERIALS AND MATERIALS

Cell-associated viral antigen

Baby hamster kidney cells (BHK-21/69) grown in 850 cm² roller bottles (Corning, Elmira, New York 14902, USA) were infected with either cell culture-adapted BTV serotype 11 (BTV-11) or EHDV serotype 2 (EHDV-2) (USDA-ARS, Laramie, Wyoming 82071, USA) at 0.024 plaque forming units per cell. Cells were harvested after 3 days (near maximum cytopathic effect) by centrifugation at 1,000 *g* for 15 min. Cell-associated antigens were prepared essentially as described by Jochim (1976). Briefly, cells were washed, harvested, twice suspended in tris buffer (0.002 M, pH 8.8), sonicated in an ice water bath and centrifuged at 2,300 *g*. The antigen protocol was modified at this point in that the resulting supernatants were pooled, twice vortexed with Fluorocarbon 113 (⅓ volume), and centrifuged at 1,000 *g* to eliminate cellular lipids. Aqueous phases were pooled and assayed for protein concentration (Bio-Rad, Richmond, California 94804, USA). For the BHK

control antigen, uninfected cell monolayers were scraped from roller bottles, then treated the same as the BTV and EHDV infected cells.

Pronghorn horseradish peroxidase (HRP)-conjugated antibody

Pronghorn serum was precipitated with saturated $(NH_4)_2SO_4$ and IgG purified on a Sephacel anion exchange column (Pharmacia, Piscataway, New Jersey 08855, USA). Rabbits were inoculated with 200 μg pronghorn IgG in complete Freund's adjuvant (50 μg subcutaneously, 50 μg intravenously, and 100 μg intramuscularly). Twenty-one and 28 days later, 200 μg IgG boosters, with adjuvant (Tetronic 1501 Polyol, BASF Wyandotte Corporation Industrial Chemical Group, Wyandotte, Michigan 49192, USA) (Hunter and Bennett, 1984; Woodard, 1989), were injected subcutaneously. After 7 days rabbits were bled and rabbit anti-pronghorn IgG was precipitated with saturated $(NH_4)_2SO_4$ (Hebert et al., 1973). Antibody was conjugated to horseradish peroxidase using the sodium m-periodate method (Nakane and Kawaoi, 1979).

Pronghorn serologic survey

One hundred sixty-one trapped or hunter-killed pronghorn serum samples from three counties in Wyoming (USA) (Johnson, Converse, and Carbon), provided by the Wyoming Game and Fish Department (Laramie, Wyoming 82070, USA), were tested with the ELISA using BTV, EHDV and BHK antigens. Bluetongue immunodiffusion assays (Pearson and Jochim, 1979) were performed by the USDA-APHIS at the Wyoming State Veterinary Laboratory (Laramie, Wyoming 82070, USA).

Sera that were BTID positive, but BTV ELISA negative were tested for the presence of EHDV antibody with the ELISA using EHDV CA antigen. Sera that were BTID negative and BTV ELISA positive were radioimmune precipitated to demonstrate serum antibodies to BT structural and non-structural viral proteins.

BTV, EHDV and BHK ELISA

Ninety-six well microtiter plates (Dynatech Immulon II, Chantilly, Virginia 22021, USA) were coated with the appropriate CA antigen (BTV-11 at 4 μg, EHDV-2 at 8 μg or BHK at 4 μg) in 100 μl coating buffer (0.07 M $NaHCO_3$, 0.03 M Na_2CO_3, pH 9.6) (Roehrig et al., 1980). Plates were allowed to stand overnight at 4 C in a humid chamber. Plates were washed three times with washing solution [phosphate buffered saline (PBS), 0.5% non-fat powdered milk (NPM), 0.05% Tween 20, 0.005% Thimerosal, pH 7.2] and rinsed once with distilled water between each of the steps. Incubation parameters were 100 μl/well, 1 hr at 37 C, unless indicated otherwise. Non-specific binding sites in the plate wells were blocked with 5% milk block solution (Swack et al., 1987) (in coating buffer, 0.005% Thimerosal), 200 μl/well, 2 hr at 37 C. Pronghorn serum samples were diluted 1:100 in dilution buffer (PBS, 0.5% NPM, 0.005% Thimerosal, pH 7.2). Twenty-two samples were tested per plate, three replicate wells/sample. Rabbit anti-pronghorn IgG-HRP conjugate (1:1,000 dilution) was then added and plates were incubated. Ortho-phenylene diamine (OPD, Hyclone Laboratories, Logan, Utah 84321, USA) (Shaw et al., 1986) was added and incubated in the dark, at room temperature for 20 min. The enzymatic reaction was stopped with 3 N sulfuric acid (50 μl/well) and the optical density (OD) was determined with a Titertek Multiskan Plus MK II (Flow Laboratories, McLean, Virginia 22102, USA) plate reader at $A_{492\ nm}$.

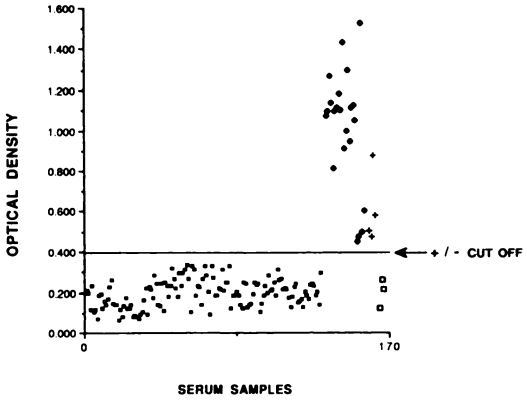

FIGURE 1. Scatter graph of pronghorn serum samples tested for antibody to bluetongue virus (BTV) with the BTV enzyme-linked immunosorbent assay (ELISA) and the bluetongue immunodiffusion assay (BTID). BTID+/ELISA+ (◆) indicate samples that tested positive for BTV antibody in both the BTID and the ELISA. BTID−/ELISA+ (+) indicate samples that were negative in the BTID, but positive in the ELISA. BTID+/ELISA− (□) indicate samples that were positive in the BTID, but negative in the ELISA. BTID−/ELISA− (■) indicate samples that were negative in both the BTID and the ELISA.

Pronghorn sera were tested with a BHK control CA antigen to determine the background reactivity of the BTV ELISA to non-viral antigen. BHK control ELISA results were subtracted from the BTV ELISA results. One hundred thirty-two BTID negative sera were used to calculate the positive/negative cutoff. Antelope sera that were BTID positive/BTV ELISA negative and two serum samples that were BTID strong positive/BTV ELISA weak positive were tested for EHDV antibody with the EHDV ELISA.

TABLE 1. Results of 161 pronghorn serum samples as tested by the enzyme-linked immunosorbent assay (ELISA) and the bluetongue immunodiffusion assay (BTID).

	ELISA		
BTID	(+)	(−)	Total
(+)	22	3	25
(−)	4	132	136
Total	26	135	161

ELISA

Sensitivity = 22/25 = 88%
Specificity = 132/136 = 97%
Predictive (+) value = 22/26 = 84.6%
Predictive (−) value = 132/135 = 97.8%

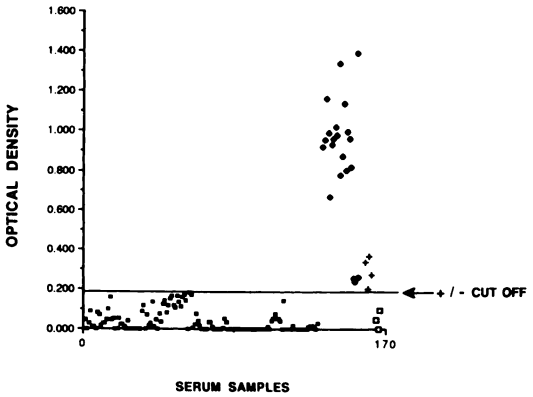

FIGURE 2. Scatter graph of pronghorn serum samples tested for antibody to bluetongue virus (BTV) with the BTV enzyme-linked immunosorbent assay (ELISA) and the bluetongue immunodiffusion assay (BTID), minus the cellular background inherent in the cell-associated antigen. BTID+/ELISA+ (◆) indicate samples that tested positive for BTV antibody in both the BTID and the ELISA. BTID−/ELISA+ (+) indicate samples that were negative in the BTID, but positive in the ELISA. BTID+/ELISA− (□) indicate samples that were positive in the BTID, but negative in the ELISA. BTID−/ELISA− (■) indicate samples that were negative in both the BTID and the ELISA.

Radioimmune precipitation

BHK-21 cells were infected with BTV-11, labelled with [^{35}S]methionine (Du Pont, NEN, Wilmington, Delaware 19801, USA), and lysed as described by Mecham et al. (1986). Viral proteins in the cell lysates were immune precipitated using pronghorn antisera in combination with *Staphylococcus aureus* protein A (Pansorbin, Calbiochem, La Jolla, California 92037, USA) as the solid phase. Immune precipitated viral antigens were analyzed by SDS-PAGE (polyacrylamide gel electrophoresis) and autoradiography.

RESULTS

ELISA

Variability in the OD results of BTID negative pronghorn test sera was seen, as well as a break in the results near 0.350 optical density (OD) (Fig. 1). The lower OD values were used to calculate a positive/negative cut off: mean OD + 3 standard deviations [0.195 + (3)0.067 = 0.396]. Test sera with an OD ≤0.396 were considered negative and sera with an OD >0.396 were considered positive.

One hundred thirty-two sera were BTID negative/BTV ELISA negative. Twenty-two sera were BTID positive/BTV ELISA positive. Four sera were BTID negative/BTV ELISA positive, and three sera were BTID positive, but consistently tested negative in the BTV ELISA (Table 1).

The OD results of the 161 antelope samples tested in the BHK control ELISA varied from 0.062 to 0.381. Results of the BTV ELISA on the 161 serum samples after subtraction of this BHK control background and calculation of the positive/negative cut off [mean OD + 3 standard deviations (0.035 + (3)0.049 = 0.182)] were equivalent to the results before subtraction of the BHK background (Fig. 2).

Three known BTID negative pronghorn serum samples, three OD readings/sample, were used for the calculation of a positive/negative cut off of 0.119 [0.095 + (3)0.008 = 0.119] for the EHDV ELISA. One of the three BTID positive/BTV ELISA negative samples was positive for EHDV antibody, and the BTID strong positive/BTV ELISA weak positive samples were both positive for EHDV antibodies by the EHDV ELISA (Fig. 3).

The 1988 estimated postharvest wintering pronghorn population in Wyoming was 363,487 animals (Wyoming Game and Fish, 1988). Of 161 antelope samples from

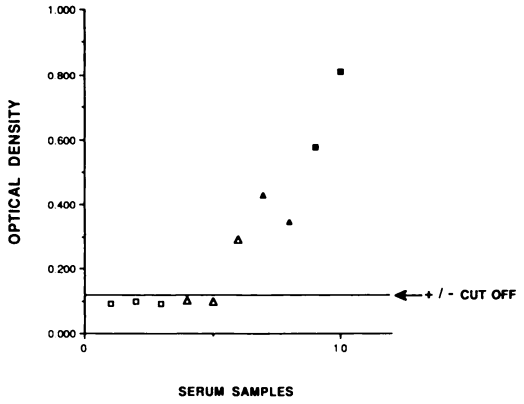

FIGURE 3. Scatter graph of pronghorn serum samples tested for antibody to epizootic hemorrhagic disease virus (EHDV) with an EHDV enzyme-linked immunosorbent assay (ELISA). BTID++/ELISA+/− (▲) indicate samples that were strong positives in the BTID, but weak positives in the ELISA. BTID+/ELISA− (△) indicate samples that were positive for BTV antibody in the BTID, but negative in the ELISA. Three known EHDV negative control antisera (□) and two known EHDV positive control antisera (■) are also shown.

three Wyoming counties, 26 samples (16%) were positive for BTV antibody with the BTV ELISA.

Radioimmune precipitation

The four discrepant BTID negative/BTV ELISA positive serum samples were demonstrated to have BTV antibodies which immune precipitated structural and nonstructural proteins of BTV (Mecham et al., 1986) (Fig. 4). The three discrepant BTID positive/BTV ELISA negative serum samples were demonstrated to be negative for antibodies to structural viral proteins (Fig. 5). Non-specific sticking of the nonstructural protein NS1 was seen in these sera as well as in a known negative control sera.

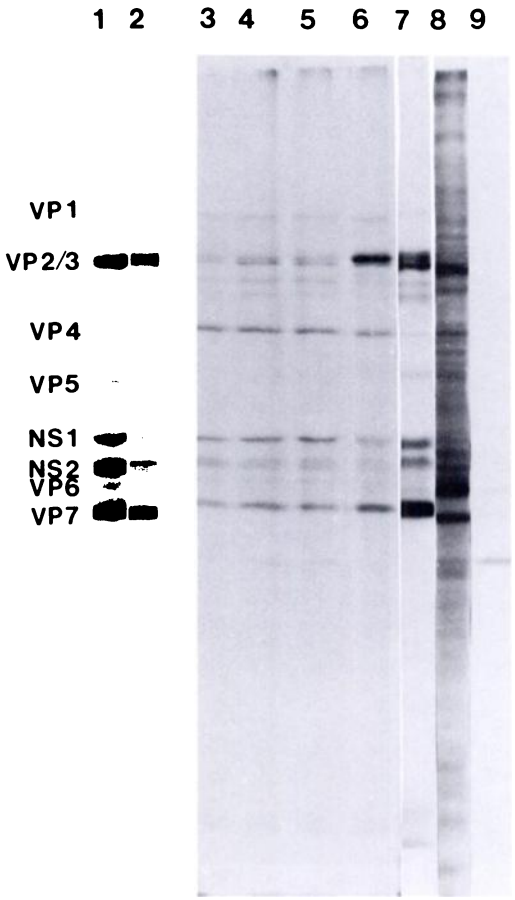

FIGURE 4. Radioimmune precipitation of cell-associated BTV-11 antigen with test sera. Viral proteins from infected cells, labelled with [^{35}S]methionine, were immune precipitated with: lane 1, rabbit antiserum to BTV-11; lane 2, bovine antiserum to BTV-11; lanes 3 to 6, BTV ELISA weak positive/BTID negative pronghorn sera; lane 7, pronghorn antiserum to BTV; lane 8, uninfected labelled cell lysate; lane 9, normal pronghorn serum.

DISCUSSION

The results of the BTID, which requires whole sera, and ELISA, which tests samples at a 1:100 dilution, were equivalent except for seven serum samples. Four BTID negative sera were demonstrated to be positive for antibodies to BTV structural and non-structural proteins by BTV ELISA and radioimmune precipitation. Three BTID positive sera were demonstrated to be negative for antibodies to BTV proteins by ELISA and radioimmune precipitation. One of the three sera was shown to be positive for EHDV antibody by EHDV ELISA, which would account for a false positive result in the BTID. The other two sera may indicate non-specific reactions in the BTID, or cross-reactive antibodies to viruses other than EHDV.

The two sera that were strong BTID

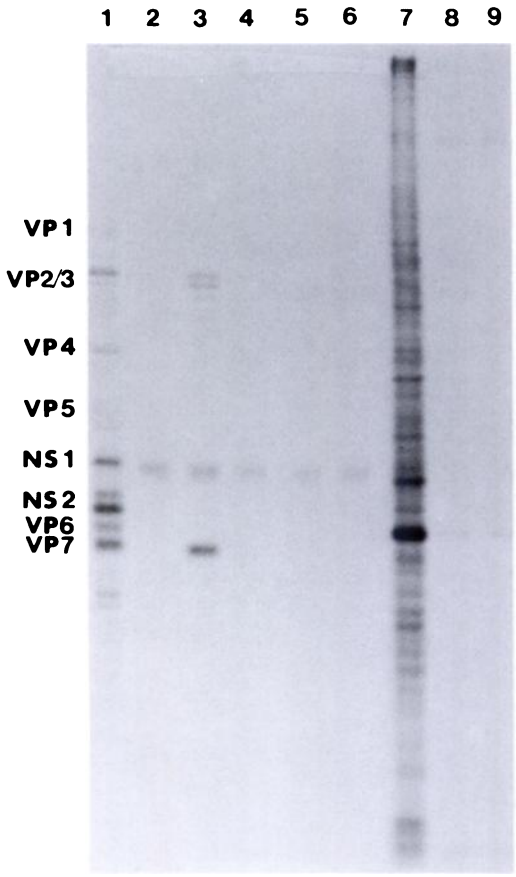

FIGURE 5. Radioimmune precipitation of cell-associated BTV-11 antigen with test sera. Viral proteins from infected cells, labelled with [^{35}S]methionine, were immune precipitated: lane 1, BTV-11 infected, labelled cell lysate; lane 2, infected lysate with known negative control pronghorn sera; lane 3, infected lysate with known positive control pronghorn sera; lane 4 to 6, infected lysate with BTID positive/BTV ELISA negative pronghorn sera; lane 7, uninfected, labelled cell lysate; lane 8, uninfected lysate with known negative control pronghorn sera; lane 9, uninfected lysate with known positive control pronghorn sera.

positives, but weak BTV ELISA positives, were shown to have EHDV antibodies by EHDV ELISA, as suspected. An animal infected with both BTV and EHDV would give a stronger positive reading by BTID which is affected by EHDV cross-reactive antibodies, than it would in the ELISA which specifically detects BTV antibodies only.

The reason the BTV ELISA is not affected by the presence of EHDV antibodies may be due to the slightly altered reactivity of cross-reactive antibodies to antigen adsorbed to polystyrene (Butler et al., 1986; Dierks et al., 1986). Another explanation is that the cross-reacting antibodies causing precipitation in the BTID are possible low-affinity antibodies which would not be detected in the ELISA (Butler et al., 1978).

Four additional positive samples along with one false BTID positive sample (EHDV positive) were discovered with the BTV ELISA, while two samples may have been false negatives in the ELISA, representing an improvement over the BTID.

Cellular background reaction in the CA antigen had negligible effects on the results when a positive/negative cut off was calculated with a known negative OD mean + 3 standard deviations to give a 99% confidence interval. The CA antigen preparation is simple, economic and high-yielding.

The increased number of samples the ELISA can test in one day compared to the BTID is substantial. Twenty-two samples, three replicate wells/sample can be run on a pre-coated plate in about 5 hr. Pre-coated plates can be stored at 4 C in a humid chamber for up to 10 wk. Several plates can be run simultaneously and semi-mechanization is possible, which lends itself well to testing large numbers of samples relatively quickly. The BTID can only test three samples per assay and must be incubated 24 hr before reading. The pronghorn BTV ELISA was adapted by this laboratory from a bovine BTV ELISA by the substitution of one reagent—the anti-species IgG-HRP conjugate. This ELISA also has been adapted to deer and elk by another laboratory with the appropriate anti-species conjugate substitution. It is probable that the ELISA could easily be adapted to moose, bighorn sheep and other wildlife.

The quantity and quality of serum samples obtained from wildlife are often less than optimal. The BTV ELISA requires only 3 μl of serum for testing as described

herein, and will give accurate results regardless of prior improper handling or storage of the sample. The BTV ELISA gave rapid, quantitative, objective results and should prove useful in testing large numbers of sera for BT diagnostic and seroepizootiologic studies.

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LITERATURE CITED

AFSHAR, A. F. C., P. F. THOMAS, P. F. WRIGHT, J. L. SHAPIRO, P. T. SHETTIGARA, AND J. ANDERSON. 1987. Comparison of competitive and indirect enzyme-linked immunosorbent assay for detection of bluetongue virus antibodies in serum and whole blood. Journal of Clinical Microbiology 25: 1705–1710.

ANDERSON, J. 1985. Monoclonal antibodies and bluetongue virus diagnosis. *In* Bluetongue and related orbiviruses, T. L. Barber and M. M. Jochim (eds.). Alan R. Liss, Inc., New York, New York, pp. 497–504.

BUTLER, J. E., T. L. FELDBUSH, P. L. MCGIVERN, AND N. STEWART. 1978 The enzyme-linked immunosorbent assay (ELISA): A measure of antibody concentration or affinity? Immunochemistry 15: 131–136.

———, J. E. SPRADLING, M. SUTER, S. E. DIERKS, H. HEYERMANN, AND J. H. PETERMAN. 1986. The immunochemistry of sandwich ELISAs-I. The binding characteristics of immunoglobulins to monoclonal and polyclonal capture antibodies adsorbed on plastic and their detection by symmetrical and asymmetrical antibody-enzyme conjugates. Molecular Immunology 23: 971–982.

DELLA-PORTA, A. J., I. M. PARSONSON, AND D. A. MCPHEE. 1985. Problems in the interpretation of diagnostic tests due to cross-reactions between orbiviruses and broad serological responses in animals. *In* Bluetongue and related orbiviruses, T. L. Barber and M. M. Jochim (eds.). Alan R. Liss, Inc., New York, New York, pp. 445–453.

DIERKS, S. E., J. E. BUTLER, AND H. B. RICHERSON. 1986. Altered recognition of surface-bound antibodies in the ELISA. Molecular Immunology 23: 403–411.

HEBERT, G. A., P. L. PELHAM, AND B. PITTMAN. 1973. Determination of the optimal ammonium sulfate concentration for the fractionation of rabbit, sheep, horse, and goat antisera. Applied Microbiology 25: 26–36.

HUNTER, R. L., AND B. BENNETT. 1984. The adjuvant activity of nonionic block polymer surfactants. II. Antibody formation and inflammation related to the structure of triblock and octablock copolymers. Journal of Immunology 133: 3167–3175.

JOCHIM, M. M. 1976. Improvement of the AGP test for bluetongue. Proceedings of the American Association of Veterinary Laboratory Diagnosticians 19: 361–376.

———. 1985. An overview of diagnostics for bluetongue. *In* Bluetongue and related orbiviruses, T. L. Barber and M. M. Jochim (eds.). Alan R. Liss, Inc., New York, New York, pp. 423–433.

MECHAM, J. O., V. C. DEAN, AND M. M. JOCHIM. 1986. Correlation of serotype specificity and protein structure of the five serotypes of bluetongue virus. Journal of General Virology 67: 2617–2624.

NAKANE, P. K., AND A. KAWAOI. 1979. Peroxidase-labeled antibody: A new method of conjugation. Journal of Histochemistry and Cytochemistry 22: 1084–1091.

PEARSON, J. E., AND M. M. JOCHIM. 1979. Protocol for the immunodiffusion test for bluetongue. Proceedings of the American Association of Veterinary Laboratory Diagnosticians 22: 463–471.

POLI, G., J. L. STOTT, Y. S. LUI, AND J. S. MANNING. 1982. Bluetongue virus: Comparative evaluation of enzyme-linked immunosorbent assay, immunodiffusion, and serum neutralization for detection of viral antibodies. Journal of Clinical Microbiology 15: 159–162.

ROEHRIG, J. T., J. A. CORSER, AND M. J. SCHLESINGER. 1980. Isolation and characterization of hybrid cell lines producing monoclonal antibodies directed against the structural proteins of sindbis virus. Virology 101: 41–49.

SHAW, R. D., P. T. VO, P. A. OFFIT, B. S. COULSON, AND H. B. GREENBERG. 1986. Antigenic mapping of the surface proteins of rhesus rotavirus. Virology 155: 434–451.

SWACK, J. A., T. NAKATSUJI, K. ITO, AND K. TSUJI. 1987. Preservation of membrane glycoprotein labile epitopes during western blotting with monoclonal antibodies. Biotechniques 5: 564–571.

THORNE, E. T., E. S. WILLIAMS, T. R. SPRAKER, W. HELMS, AND T. SEGERSTROM. 1988. Bluetongue in free-ranging pronghorn antelope (*Antilocapra americana*) in Wyoming: 1976 and 1984. Journal of Wildlife Diseases 24: 113–119.

TRAINER, D. O., AND M. M. JOCHIM. 1969. Serologic evidence of bluetongue in wild ruminants of North America. American Journal of Veterinary Research 30: 2007–2011.

WHITE, J. R., A. J. DELLA-PORTA, AND D. A. MCPHEE. 1985. Serological diagnosis of orbivirus infections of livestock. *In* Veterinary viral dis-

eases: Their significance in south-east Asia and the western Pacific. Proceedings of the Australian National Animal Health Laboratory, CSIRO, Geelong, Australia, A. J. Della-Porta (ed.). Academic Press, Sydney, Australia, pp. 426–429.

WOODARD, L. F. 1989. Adjuvant activity of water-insoluble surfactants. Laboratory Animal Science 39: In press.

WYOMING GAME AND FISH. 1988. Pronghorn antelope. *In* The 1988 annual report. Wyoming Game and Fish Department, Cheyenne, Wyoming, p. 16.

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