Development and Evaluation of an Enzyme-linked Immunosorbent Assay for Detection of Bovine Antibodies to Epizootic Hemorrhagic Disease of Deer Viruses

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ABSTRACT

An indirect enzyme-linked immunosorbent assay (I.ELISA) is described for detection of bovine serum antibody to epizootic hemorrhagic diseases of deer virus (EHDV). Serum samples, at a dilution of 1:200, were incubated with group-specific EHDV antigens, pre-adsorbed to microtiter plates. Bound antibodies were detected by a murine monoclonal antibody to bovine immunoglobulin (Ig)G1 (heavy-chain specific) conjugated with horseradish peroxidase. The performance of the I.ELISA in detecting antibodies to EHDV in sequential serum samples from calves experimentally infected with serotypes 1,2,3 and 4 was evaluated. The I.ELISA detected EHDV antibodies from 14 days postinfection when seroconversion by the standard agar gel immunodiffusion (AGID) test was also evident. The group-specific antibodies to EHDV increased exponentially during the first two to four weeks postinfection and remained relatively stable for about 12 months in some calves. Unlike observations with the AGID test, no reaction was seen in the I.ELISA between bluetongue virus (BTV) antigen and sera from calves given a single dose of EHDV. The performance of the I.ELISA and AGID were compared using 3,135 AGID negative bovine field sera from herds in Ontario, Alberta and British Columbia and 130 AGID positive samples collected from cattle in 1987 and 1988 during and after outbreaks of EHD in the Okanagan Valley, British Columbia. The specificity and sensitivity of the assay relative to the AGID test were 99.3% and 91.5% respectively, with an overall agreement of 99.0% between the tests. The results suggest that the I.ELISA offers many advantages over the AGID and has potential application as a rapid and inexpensive test in serodiagnosis of epizootic hemorrhagic disease virus infections in cattle.

RÉSUMÉ

Une méthode immunoenzymatique indirecte (ELISA-I) pouvant déceler la présence d'anticorps bovins contre le virus de la maladie hémorrhagique du cerf (MHC) est décrite. L'antigène de la MHC fut d'abord adsorbé sur microplaques et les échantillons de sérum, dilués 1:200, furent mis en contact avec l'antigène. Les anticorps fixés à l'antigène de la MHC furent décelés au moyen d'un anticorps monoclonal murin conjugué à la peroxydase de raifort. Ce dernier anticorps possède une spécificité pour la chaîne lourde de l'immunoglobuline (Ig) bovine IgG1. L'efficacité de l'épreuve d'ELISA-I à déceler des anticorps bovins à la suite d'une infection expérimentale avec le virus de la MHC types 1, 2, 3 et 4 fut évaluée. L'épreuve d'ELISA-I a décelé des anticorps contre le virus MHC aussitôt que 14 jours après l'infection. Les anticorps de groupe contre le virus de la MHC ont augmenté de façon exponentielle durant les premières 2 à 4 semaines et, chez certains veaux, leur niveau est demeuré stable durant 12 mois. Contrairement à ce qui fut observé avec l'épreuve d'immunodiffusion (ID), aucune réaction ne fut décelée par l'épreuve d'ELISA-I avec l'antigène du virus de la fièvre catarrhale bovine et les sérums des veaux qui ont reçu une seule dose du virus de la MHC. L'efficacité de l'épreuve d'ELISA-I fut comparée à celle de l'ID sur 3,135 échantillons de sérum préalablement classifiés négatifs en ID. Ces échantillons provenaient de troupeaux de l'Ontario, de l'Alberta et de la Colombie-Britannique (C-B). La comparaison fut aussi effectuée sur 130 sérums positifs en ID. Ces derniers sérums provenaient de bovins échantillonnés durant et après les épizooties de la MHC qui ont sévi en C-B en 1987 et 1988. La spécificité et la sensibilité de l'épreuve d'ELISA-I comparativement à celle de l'ID furent respectivement de 99.3% et de 91.5%. Une concordance de 99.0% fut observée entre les deux épreuves. Parce que l'ELISA-I est rapide et économique, elle offre un potentiel plus intéressant que l'épreuve d'ID dans le diagnostic sérologique d'une infection par le virus de la MHC chez le bovin.

INTRODUCTION

The virus causing epizootic hemorrhagic disease of deer (EHD) was first identified in white-tailed deer in the United States (1,2). The isolation of related viruses from wild and domestic ruminants has been reported from Canada (3,4), Japan (5), Korea (6), the United States (7–9) and Australia (10,11). Along with bluetongue (BT) and African horse sickness viruses, EHD viruses are classified in the Orbivirus genus of the Reoviridae family

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(12,13). Two serotypes designated as EHDV type 1 (T-1) (New Jersey) and type 2 (T-2) (Alberta), serologically related to but distinct from BTV, are enzootic in the United States. Similar to the first report of EHD in Canada (3), the recent incursion of EHD into the Okanagan Valley, British Columbia was caused by EHDV, T-2 (4). The EHDV T-1 and T-2 infections have rarely been associated with overt clinical disease in domesticated cattle and sheep under natural field conditions and reports of experimental clinical EHD are controversial (10,14–17).

Epizootic hemorrhagic disease virus infections in domestic and wild ruminants are most often detected by the presence of anti-EHDV antibodies. Several tests are described in the literature that detect group-specific or serotype-specific antibodies (18–20). The agar gel immunodiffusion (AGID) test is the most widely used assay. Although it is simple and rapid to perform, the AGID may be difficult to interpret, may lack sensitivity, is not quantitative and may be complicated by cross-reactions with other orbiviruses, e.g. BTV (21–23). As an alternative, indirect enzyme-linked immunosorbent assays (I.ELISA) have been developed to detect anti-EHDV antibodies (24,25). Since there are no reports on the large scale application and validation of these assays for serodiagnosis of EHDV infections, we decided to develop and compare the performance of an I.ELISA and the standard AGID for detection of anti-EHDV T-1 and T-2 antibodies. The present report summarizes our experience with the I.ELISA in the testing of sera collected from cattle experimentally infected with EHDV and field sera from cattle in Canada.

MATERIALS AND METHODS

TISSUE CULTURE AND EHD VIRUSES

Monolayer cultures of baby hamster kidney (BHK-21) cells were grown in equal volumes of Earle's minimum essential medium (MEM) and L15 medium (Gibco Laboratories, Grand Island, New York) supplemented with 1% L-glutamine, 10% tryptose phosphate broth, 10% fetal bovine serum (FBS), free of bovine viral diarrhea virus (BVDV) and anti-BVDV antibodies, and antibiotics. Following a wash with 0.05 M phosphate buffered saline (PBS), pH 7.4, each bottle was inoculated with 25 mL of plaque-purified EHDV diluted in MEM to contain $10^{6.3}$ median tissue culture infectious dose ($TCID_{50}$), giving a multiplicity of infection of approximately 0.01. The New Jersey strain of EHDV T-1 and the Alberta strain of EHDV T-2, both originally obtained from Dr. J.E. Pearson, National Veterinary Services Laboratories, Ames, Iowa were used in this study. After an adsorption period of 60 min at 37°C, the cultures were maintained in MEM without FBS and rolled at 0.25 rmp. When an 80 to 90% cytopathic effect developed, approximately 90 h after inoculation, the cultures were harvested by freezing at −70°C and thawing at 37°C.

EHDV ANTIGEN PREPARATION

Lots of antigen for the I.ELISA were prepared according to a modified procedure originally described for a bluetongue ELISA by Anderson in 1984 (26). Briefly, the harvested virus suspension was centrifuged at 3,000 × *g* for 30 min at 4°C. The cell pellet was resuspended in 1:100th the original tissue culture volume in 0.002 M Tris/HCl lysis buffer, pH 8.4 containing 1% Triton-X100, 0.2 M KCl, 0.1 M $MgCl_2$ and 0.0072 M $CaCl_2$ and homogenized in a glass homogenizer (Tenbroeck). The nuclei were then pelleted by centrifugation at 800 × *g* for 10 min, resuspended in the same lysis buffer, pelleted once more and then discarded. Following the addition of heparin (250 units/mL), the supernatant pool, representing cytoplasmic extracts, was sonicated on ice with four 60 s bursts using an ultrasonic disrupter (Model W375, Branson Ultrasonic Corporation Canada, Scarborough, Ontario) with a 0.5 inch probe and a 60% amplitude setting. This solubilized virus preparation was layered on a 40% (W/V in Tris/HCl, pH 8.4 buffer) sucrose cushion and centrifuged at 100,000 × *g* for 2 h at 4°C in an ultracentrifuge (RC 80, Sorvall Instrument, Dupont Co., Wilmington, Delaware). The resulting pellet was resuspended in 1:600th the original tissue culture volume in Tris/HCl, pH 8.4, without Triton, homogenized by pulse sonication and along with a similar preparation from uninfected BHK-21 cells, was used as ELISA antigen in this study.

SERUM SAMPLES

Sera from serial bleedings of groups of calves experimentally infected and/or challenged with different serotypes of EHDV were studied (Table I). Sera collected from calves in Groups I, II and III were obtained from other laboratories (23,28). Two calves in group IV were inoculated and

TABLE I. Bovine sera raised to some EHDV and BTV types

Group	Calf No.	Virus inoculum		Source (reference)
		Primary	Secondary	
I	C.7953	EHDV-1	EHDV-2	Bowen, R.A. (28)
	C.7954	"	"	"
	C.7957	"	"	"
II	C.7955	EHDV-2	EHDV-1	"
	C.7956	"	"	"
III	OW.17	EHDV-1	ND	Taylor, W.P. (23)
	OW.18	EHDV-2	"	"
	OC.92	EHDV-3	"	"
	OC.94	"	"	"
	OC.89	EHDV-4	"	"
	OC.93	"	"	"
IV	C1.86	EHDV-2[a]	EHDV-2[a]	Myers, D.J. (ADRI)
	C2.86	"	"	"
	C14.73	EHDV-2[b]	EHDV-2[b]	Ruckerbauer, G. (ADRI)
	C15.73	"	"	"
V	C4.85	BTV-10	ND	Thomas, F.C. (27)
	C	BTV-11	"	"
	C	BTV-13	"	"
	C	BTV-17	"	"

[a]Suckling mouse brain virus
[b]Cell culture (BHK-21, W1-2 subline) virus
ND: Not done
ADRI: Animal Disease Research Institute, Nepean

challenged three weeks later by combined intravenous, subcutaneous and intramuscular routes with a suckling mouse brain (SMB) stock of EHDV T-2. Each calf received approximately $10^{7.6}$ $TCID_{50}$ of the virus on the primary and secondary inoculation days. Both calves subsequently received SMB-derived EHDV T-1 four weeks after the challenge day. Similarly, two other calves in group IV were inoculated initially and challented four weeks later with EHDV T-2 grown in the WI-2 subline of BHK-cells (29).

A total of 3,135 EHD and BT AGID negative field sera were used to evaluate the specificity of the I.ELISA and to define a negative cut-off value for the Canadian cattle population. These sera consisted of 897 samples from dairy herds in Ontario and 396 samples from beef cattle in Alberta, both collected in 1986. Furthermore, panels of 888 and 954 bovine sera, collected in 1987 from British Columbia and southwestern Alberta respectively, for surveillance of EHD and BT (31) were included in this study. One hundred and thirty cattle sera positive in the AGID for EHDV were also included for evaluation of the relative sensitivity of the I.ELISA.

ELISA PROCEDURE

The I.ELISA was based on a previously described procedure (27,33), except that a murine monoclonal antibody (MAb) to bovine immunoglobulin (Ig)G1 (heavy-chain specific) conjugated with horseradish peroxidase (HRP) (33) was used to detect bound bovine antibody. In brief, flat-bottomed 96-well polystyrene plates (Linbro, Titertek, Flow Laboratories, Mississauga, Ontario, Cat # 76-301-05) were optimally coated by passive adsorption with 100 μL/well of a mixture of EHDV T-1 and T-2 stock antigens or control cell antigen, at a dilution of 1:200 in 0.06 M carbonate buffer, pH 9.6. After overnight incubation at 25°C the wells were washed four times with 0.01 M PBS, pH 7.2 containing 0.5% Tween-20 (PBST). Serum samples were tested at a single dilution of 1:200 in PBST containing 1% gelatin (Bio-Rad, Richmond, California; Cat # 170-6537), and 100 μL samples were applied in duplicate in a diagonal sample placement pattern (34). Following incubation for 3 h at 25°C, the wells were again washed and filled with 100 μL of 1:4,000 HRP-MAb conjugate (M23 Lot #5) in PBST. After incubation for 60 min at 25°C, the conjugate solution was discarded and the plates were again washed in PBST. The wells were filled with 100 μL of substrate solution containing 2.0 mM ABTS [2,2-azino-di-(3-ethylbenzthiazoline-sulfonic acid)] and 4 mM H_2O_2 in 0.05 M citrate buffer, pH 4.2, and the plates were then shaken continuously at 25°C for approximately 10 min. Using a computer-assisted target timing protocol (35), the plates were incubated until the strong positive control well chromogen development reached an optical density (OD) of 1.0. Chromogen conversion was measured at 414 nm with a Titertek Multiskan MC microplate photometer (Flow Laboratories) which had been previously blanked on 100 μL substrate/chromogen in a separate microtiter plate which had been pre-rinsed with PBST. Strong and weak positive sera to EHDV T-1 and EHDV T-2 and PBST were included in each microtiter plate as quality controls. For the purpose of evaluating antibody profiles, OD values for sera from the experimentally infected calves were expressed as the mean OD value resulting from the reaction with the viral antigen minus the reaction with the cell control antigen. Field serum samples were considered negative, suspicious and positive if their P/N ratios (viral antigen OD value over control antigen OD value) were 2.0 or lower, between 2.01 and 2.50 and 2.51 or greater, respectively.

AGID TEST

The AGID test was the standard method described by Pearson and Jochim (36).

RESULTS

In order to confirm the usefulness of the HRP conjugated MAb (M23) in the EHD I.ELISA, sera from experimentally exposed calves were tested. The I.ELISA results compared favourably with those of the commercial HRP labelled rabbit antibovine IgG (H and L-chain) (Cooper Biomedical, Malvern, Pennsylvania, Lot #26936) (Fig. 1). Hence, the HRP labelled MAb to bovine IgG (M23) was used throughout this study.

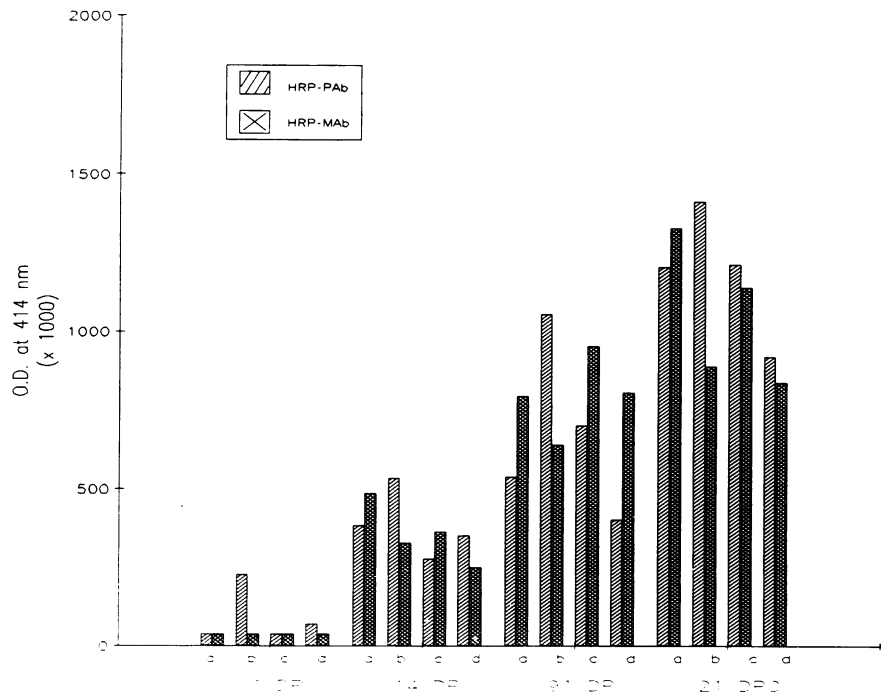

Fig. 1. Comparative antibody determinations on sera from four individual calves (a–d) infected and challenged with EHDV T-1 and/or T-2 in an I.ELISA with either a monoclonal antibovine IgG1 (H-chain) antibody (MAb) or a rabbit antibovine IgG (H and L Chain) antibody (PAb) both conjugated with horseradish peroxidase (HRP). DPI = Day postinfection; DPC = Day postchallenge.

The humoral antibody profiles as measured by the I.ELISA and AGID test in calves experimentally infected with various serotypes of EHDV are shown in Figs. 2–4. The I.ELISA detected EHDV antibodies from 14 days postinfection when seroconversion by AGID test was also evident (Fig. 2). The I.ELISA group-specific antibodies to EHDV increased exponentially during the first two to four weeks postinfection (Figs. 2 and 3) before becoming relatively stable at elevated levels for the duration of 16 weeks for calves in group I and II and 12 months for animals in group III (Fig. 4). After challenge exposure with EHDV, calves responsed anamnestically to EHDV as demonstrated by the I.ELISA (Fig. 3). Unlike the AGID the I.ELISA was shown to be group-specific for the detection of anti-EHDV antibodies in calves exposed to a single dose of EHDV. No cross-reaction with bluetongue group-specific viral antigen, as measured by another indirect ELISA (27) was demonstrable in sera from calves infected with EHDV serotypes 1,2,3 and 4 (Fig. 4). However, as observed with the AGID test following a second exposure of calves in group I, II and IV to EHDV, cross-reactions between the sera and the BTV antigen in I.ELISA were demonstrable (Fig. 2).

A total of 3,135 field sera, negative in the AGID test for antibodies to EHDV T-2 and BTV were tested in the I.ELISA for antibodies to EHDV. The frequency distribution of P/N ratios is shown in Fig. 5. Based on the cumulative data, a negative cut-off P/N ratio of 2.00 or less was selected. The frequency distribution of P/N values of 130 field bovine sera, positive in the AGID test for antibodies to EHDV T-2, are also shown in Fig. 5. A positive cut-off value of 2.51 or greater was selected arbitrarily for I.ELISA positive samples. The comparative results of the I.ELISA and AGID on the total AGID test negative and positive samples (n = 3,265) are presented in Table II. Based on the established negative and positive cut-off values, 85 samples were classified as suspicious (P/N ratios of 2.01 to 2.50). The I.ELISA test on sera diluted 1:200 demonstrated fewer positive and negative results than did the AGID test. The I.ELISA results of the suspicious sera were not included in the calculation of relative sensitivity (91.5%) and specificity (99.3%). Relative to the AGID test, a total of 30 samples gave discrepant results by the I.ELISA. Nine samples were AGID positive/I.ELISA negative and 21 were AGID negative/I.ELISA positive. The level of overall agreement between AGID and I.ELISA tests was 99.0%.

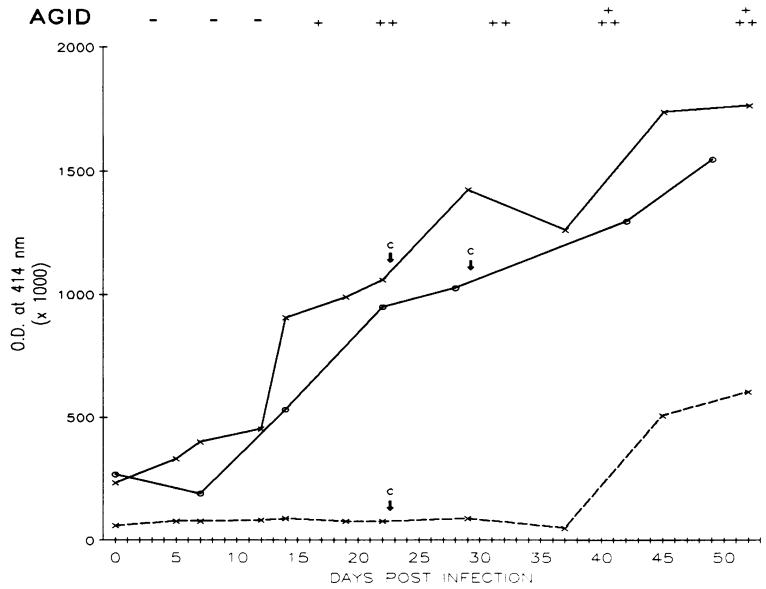

Fig. 2. Comparative development of EHDV and BTV antibodies measured by I.ELISA-EHD (———) and I.ELISA-BTV (- - - - -) and by AGID-EHD (−, negative); 1+, curved precipitating line; 2+, weak identity line; 3+, strong identity line) in serum samples from two groups of calves infected and challenged (c) with suckling mouse brain — (X———x,n = 2) and tissue culture-derived (o———o, n = 2) EHDV T-2.

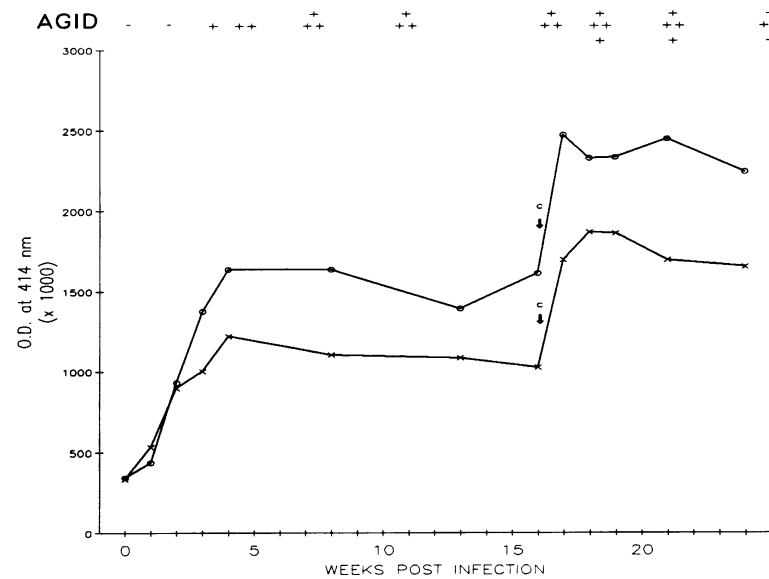

Fig. 3. Comparative development of EHDV antibody measured by I.ELISA and AGID (see legend to Fig. 2 for symbols and 4+: very strong identity line) in serum samples from two groups of calves infected with EHDV T-1 (o———o, n = 2) and EHDV T-2 (x———x, n = 3) and challenged (c) with the heterologous T-1 and T-2 of EHDV.

DISCUSSION

Despite several descriptions of ELISA methods (24,25) for the diagnosis of EHD, the AGID remains the most common method for the detection of antibodies to EHDV in cattle sera. The common antigenic determinants found on the nonstructural

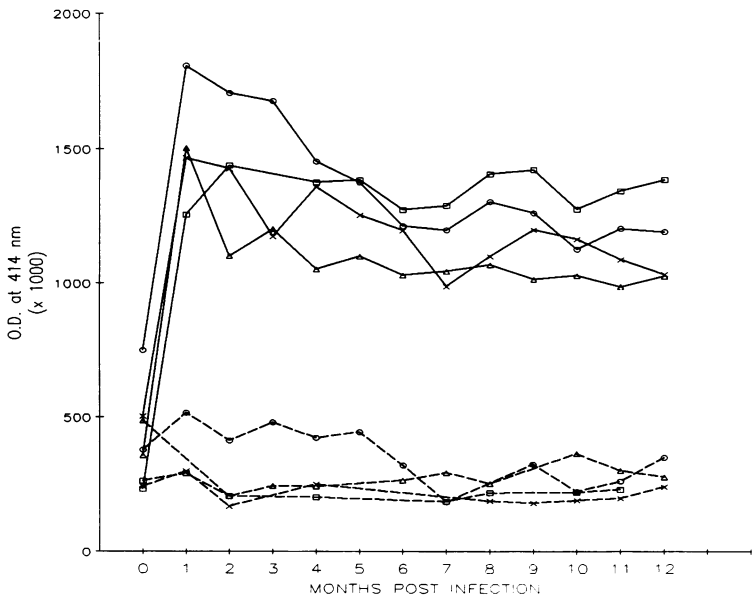

Fig. 4. Comparative development of EHDV and BTV antibodies measured by I.ELISA (———) and I.ELISA-BT (- - - -) in serum samples from calves infected with EHDV T-1 (x- - - - - - - x, n = 1), T-2 (o- - - - - - o, n = 1) T-3 (□- - - - - - □, n = 1) and T-4 (△- - - - - - △, n = 2).

and core proteins of BTV and EHDV (37) have been considered to be responsible for the complex serological (e.g. AGID) cross-reactivity reported between these and other orbiviruses (22,28,38). The method of antigen preparation reported here does not influence the specificity of the I.ELISA for the detection of antibodies to EHDV. Sera from calves given a single dose of EHDV (group IV) did not cross-react with the EHDV antigen in the bluetongue I.ELISA (27,30) (Fig. 4). Similar to BTV (27,30) the EHDV antigen preparation represents the viral core proteins and was reactive against the EHDV group antibodies in the I.ELISA. Furthermore, in order to broaden intra-group reactivity of the test serum, we combined antigens of the two North American EHDV T-1 and T-2 in a single screening I.ELISA. Any reactive serum in this screening assay can be tested by neutralization test (20) for the presence of serotype-specific antibodies.

The availability and use of the HRP-labelled MAb to bovine IgG1 facilitates standardization of the assay and reduces test variability which may be caused by different lots of commercial polyclonal enzyme conjugates. The MAb used in this study (M23) is used routinely in other ELISA tests (e.g. *Brucella* I.ELISA) at the Animal Diseases Research Institute (33).

The I.ELISA appears to be more sensitive in detecting antibodies to EHDV in cattle sera collected during the early phase after experimental infection. In three of nine calves, EHDV antibody was demonstrable as early as 10 DPI. All the experimental calves seroconverted to EHDV by day 21. Peak antibody activity was demonstrable between four to five weeks after infection in sera from calves given a single EHDV inoculation. As expected, following a second inoculation with the homologous or heterologous EHDV serotype, an anamnestic antibody response was demonstrable in sera from the experimental animals, as measured by the I.ELISA (Fig. 3). Similar to neutralizing and precipitating antibodies (14,23,25,28) anti-EHDV antibodies as measured by the I.ELISA persisted, even after a single inoculation of EHDV (group III), for up to one year (Fig. 4).

Serum screening dilutions of 1:100 and 1:200 for different EHDV antigen preparations have been used by Lunt *et al* (25) in an I.ELISA similar to our assay. We have also found that a dilution of 1:200 of serum in PBST containing 1% gelatin suitably minimizes the nonspecific background activity of antibody negative sera in the I.ELISA. At this dilution we tested several hundreds of AGID negative samples from cattle and found that approximately 1% of these sera resulted in a high background with OD values of 0.3 or greater. Based on the P/N ratio of the I.ELISA results of 3,135 AGID negative and 130 AGID positive field samples, negative and positive cut-off values of 2.0 or lower and 2.51 or greater, were established, respectively. Of the total 3,265 field sera, 85 samples (2.6%) consisting of 23 AGID positives and 62 AGID negatives, had P/N ratios between 2.01 to 2.50. Exclusive of the samples in the suspicious category, the level of agreement between the I.ELISA and the AGID test for the remaining 3,180 field sera from cattle was 99% with 21 and 9 samples classified as false positives and false negatives, respectively. Relative to AGID test results the specificity and sensitivity estimates of the I.ELISA were 99.3% and 91.5%, respectively. Five of the eight available serum samples classified as AGID positive/I.ELISA negative, neutralized EHDV T-2 when screened at a 1:20 dilution. Neutralizing antibodies to EHDV T-2 but not to EHDV T-1 were found in four of 21 serum samples classified as AGID

TABLE II. Comparison of the I.ELISA and the AGID test in the detection of EHDV antibodies in 3265 bovine field sera

	Indirect ELISA[a]					
Assay result	Pos	Neg	Susp	Relative sensitivity[b]	Relative specificity[c]	Overall agreement[d]
AGID[e]						
Pos	98	9	23	91.5%		99.0%
Neg	21	3052	62		99.3%	

[a]Sera was classified according to their P/N ratios: positive (pos), 2.51 or greater; negative (neg), 2.0 or lower; suspicious (susp), between 2.01 to 2.50

[b]Sensitivity = (I.ELISA and AGID pos/AGID pos − susp) × 100

[c]Specificity = (I.ELISA and AGID neg/AGID neg − susp) × 100

[d]Overall agreement = [(I.ELISA and AGID pos) + (ELISA and AGID neg)]/(AGID pos − susp) + (AGID neg − susp) × 100

[e]Sera giving weak to strong lines of identity with the pos reference serum were scored as pos

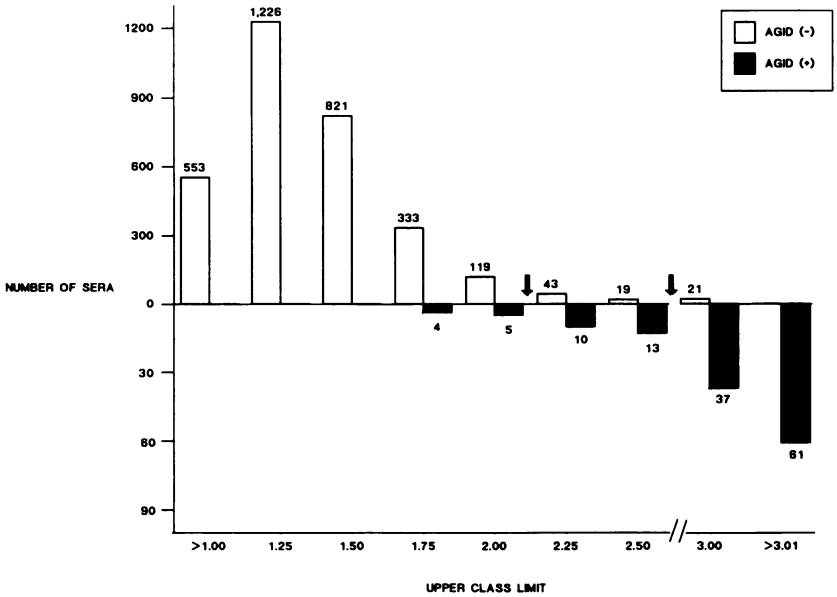

Fig. 5. Frequency distribution of P/N ratios of I.ELISA with 3135 AGID negative and 130 AGID positive bovine field sea. The arrows mark the suspicious range of P/N ratios.

negative/I.ELISA false positives. Whether the higher screening dilution of 1:200 for serum samples in the I.ELISA resulted in such discrepancy remains to be investigated. A higher sensitivity for another ELISA compared to the AGID was reported by Lunt *et al* (25) in the testing of a limited number of field sera. Further testing of a large number of EHD AGID and SN positive field sera has to be undertaken to confirm the higher sensitivity of the I.ELISA inferred by these studies.

In conclusion, it would appear that the I.ELISA may be suitable as a routine diagnostic method and may have the potential to replace the AGID test for simultaneous screening of bovine serum samples for antibodies to EHDV type 1,2,3 and 4 and possibly other serotypes. However, the I.ELISA as described here cannot be applied to testing of sera from other species, especially wild ruminants. Development of other ELISAs, e.g. competitive or blocking assays similar to those reported for the detection of antibodies to BTV (26,27,30,39,40,41) in which a group specific MAb to EHDV antigen is used, may provide a universal test for screening sera from all species.

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