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AN ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR THE DETECTION OF ANTIBODIES TO BOVINE VIRAL DIARRHOEA VIRUS (BVDV) IN CATTLE SERA

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ABSTRACT

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A microtitre ELISA has been established for the quantitation of antibodies to bovine viral diarrhoea virus (BVDV). Single dilutions of sera were assayed and units of antibody were calculated from a standard curve. In order to detect the maximum number of responding animals both IgG1 and IgG2 antibody should be assayed, although detection of IgG1 alone was nearly as effective. The ELISA was as sensitive as the virus neutralization test for detection of antibody; comparison of an ELISA that detected IgG1 plus IgG2 antibody to BVDV with the virus neutralization test gave a correlation coefficient (r) of 0.89 ($P < 0.001$ for 95 compared sera).

Although similar amounts of IgG1 and IgG2 antibodies were present in sera from both experimentally- and naturally-infected cattle, antibody to BVDV in colostrum and in the sera from young calves was predominantly IgG1. The number of adult cows with antibody was 40 out of 41 while 36 of 44 calves reared in a beef unit were found to have produced antibody by the time they were 31.5 weeks old, an indication of the high prevalence of BVDV in the cattle population.

INTRODUCTION

Serological evidence indicates that infection with bovine viral diarrhoea virus (BVDV) is very common in cattle. Estimates of immune cattle in national herds have been reported to vary from 8 to 100% (Phillip and Darbyshire, 1971; Leiss et al., 1974), while Stott et al. (1980) reported that about 50% of the beef calves they examined had developed serum antibody by the time they were 8 months old.

Infection of the foetus with BVDV can lead to abortion, foetal resorption or teratogenic defects (Van Oirshot, 1983). It can also result in the birth of calves with a persistent viraemia, and a defective immunological capacity to respond to BVDV; these animals produce little or no antibody detectable

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in the neutralization test and are the ones that are at risk from subsequently developing clinical mucosal disease (Malmquist, 1968; Leiss et al., 1974; Steck et al., 1980; Roeder and Drew, 1984). BVDV infections of normal cattle are usually acute, transient and sub-clinical (Kahrs, 1971). Although typically associated with enteric infections, BVDV has also been associated with outbreaks of respiratory disease in calves (Phillip and Darbyshire, 1971; Stott et al., 1980).

An ELISA would have several advantages over the neutralization test for detecting antibody. These include: economy of labour; more rapid availability of results; and no requirement for cells and cell culture during the assay. However, earlier studies with BVDV (Fernelius, 1966) indicated that most virus neutralizing activity was in the IgM fraction from bovine sera and there was an unusually persistent IgM antibody response following infection. Furthermore, a restricted isotype response has been reported for certain other infections of cattle (Musoke et al., 1981). Initially, therefore, an end-point titration method was used to examine the predominant isotypes of BVDV antibodies. Subsequent studies compared the end-point titration method, as the basic quantitative assay (De Savigny and Voller, 1980), with the more practical single dilution method utilising a standard curve. Further comparisons were made with the virus neutralization test for detecting antibody in various bovine sera and colostrum.

MATERIALS AND METHODS

Preparation of antigen

Monolayers of calf testis cells were prepared in 40-oz round bottles. The cultures were inoculated with $10^{7.5}$ TCD_{50} of BVDV (NADL strain) and rolled for 1 h; 20 ml of cell culture medium was then added. The medium consisted of Eagle's basal medium (Gibco Ltd.) with the addition of 2% heated (56°C, 30 min) foetal bovine serum. After incubation for 18—24 h at 36°C, by which time a marked cytopathic effect was evident, the medium was removed, and the cells suspended in 20 ml phosphate buffered saline (Dulbecco A, PBSa) by using a silicone rubber policeman. The cells were sedimented by centrifugation at 20°C for 15 min at 500× g and 2 ml of 1% Nonidet P40 (BDH Chemicals Ltd.) added to the pellet. After mixing, the cell suspension was incubated for 60 min at 37°C and aliquots stored at −70°C. As required, samples were thawed, centrifuged at 20°C for 15 min at 500 *g* and the clear supernatant removed and stored at 4°C for use as antigen. Calf testis cells that had not been infected with BVDV were extracted in the same way to provide control antigen.

Enzyme-linked immunosorbent assay (ELISA) for BVDV

The method of Voller and Bidwell (1976) was modified as follows: the Nonidet-extract of BVDV infected cells contained about 20 mg protein

ml^{-1} by the Biorad assay with ovalbumin as a standard; the extract was diluted (usually 1/50—1/100) in distilled water and extract of non-infected cells was diluted to give a similar protein concentration, i.e., 200—400 $\mu g\ ml^{-1}$. The optimum concentration of antigen was determined in the usual manner.

For the assay, alternate rows of wells in the same microtitre plate (flexible polyvinyl, Falcon 3912, Becton Dickinson, Gt. Britain) were sensitised with 50 μl of diluted extract of either BVDV-infected or non-infected cells. Plates were then incubated overnight at 37° C to dry the extract. All subsequent incubations were at room temperature, about 20° C; all plates were washed 5 times with PBSa containing 0.05% Tween 20; all dilutions of test sera, rabbit anti-bovine immunoglobulins (Ig), and enzyme-coupled goat anti-rabbit IgG were in PBSa containing 0.05% Tween 20 and 5% normal swine serum that was free from antibody to BVDV by the virus neutralization test.

Reagents were obtained from Miles Laboratories Ltd. (rabbit anti-bovine IgG1, IgG2, IgA and horse-radish peroxidase (HRP) coupled goat anti-rabbit IgG) or Nordic Immunological Laboratories Ltd. (rabbit anti-bovine IgM). Optimum dilutions for each reagent were determined. These were: anti-IgG1 1/15 000; anti-IgG2 and -IgM 1/10 000; anti-IgA and HRP anti-rabbit Ig 1/5 000. The substrate (100-μl volumes) was 40 mg O-phenylenediamine (OPD) per 100 ml of citrate-phosphate buffer pH 5 (0.025 M citric acid, 0.05 M Na_2HPO_4) containing 50 μl of 30% H_2O_2. The optical density (OD) was read at 492 nm, after the reaction had been stopped with 25 μl 2M H_2SO_4, against substrate blank on a Titerek Multiskan (Flow Laboratories).

The end-point titration assay for antibody was as follows: 200 μl of PBSa containing 0.05% Tween and 5% swine serum (diluent) was added to all wells of a sensitised plate; plates were incubated for 30 min and washed, test and control antisera were diluted in 50-μl volumes in appropriate rows of wells sensitised with BVDV or control antigen; plates were incubated for 90 min and washed; 50 μl of appropriate rabbit anti-bovine Ig sera was added per well; plates were incubated for 90 min and washed; 50 μl HRP coupled goat anti-rabbit serum was added per well; plates were incubated for 90 min and washed; 100 μl substrate was added per well; plates were incubated for 20—30 min, the reaction was stopped with 25 μl 2 M H_2SO_4 per well and the OD read at 492 nm.

A positive control serum was included in all assays for each isotype. The titre of this antiserum was obtained by comparison with known negative sera, which were taken as having titres of < 50 (De Savigny and Voller, 1980). The number of units of antibody in this standard serum was taken as being equal to the titre.

To determine the antibody levels in test sera, the corrected OD value was calculated for each dilution from the following formula: (OD in wells with BVDV antigen) — (OD in wells with control antigen), these values were plotted against serum dilutions and the end-point defined as the reciprocal

of the dilution producing the same OD value as the dilution of the standard serum that contained 1 unit of antibody.

For the single dilution assay the same method was used with only rabbit anti-bovine IgG1 and IgG2 sera. Test sera were diluted 1/100 and added to duplicate wells sensitised with BVDV or control antigen. The corrected OD (average OD in wells with BVDV antigen — average OD in wells with control antigen) was calculated and the units of antibody read off the standard curve.

Neutralization assay for antibody to BVDV

Calf kidney cells were used in a microtitre assay to measure neutralizing antibody to BVDV (Frey and Leiss, 1971). Titres (50% end-point) were recorded as the reciprocal of the highest dilution, obtained after addition of virus, that inhibited cytopathogenicity due to 10^2 TCD_{50} of strain NADL.

Bovine sera

Groups of sera from several lots of animals were examined. The first group was from 10 calves, 5 of which had been infected with a cytopathic strain of BVDV, and 5 with a non-cytopathic strain. Sera were taken before intranasal infection and 4.5 weeks after. The calves and the experimental infection have been described previously (Nuttall et al., 1980).

The second group of sera was from 41 cows from the institute's herds representing older animals that were likely to have been exposed to BVDV during their life. Colostrum was taken from 13 of these cows and tested.

The third group was from 21 calves aged 1—9 days that had been fed colostrum; 13 were fed colostrum from the cows in Group 2 above.

The fourth group of sera was from 44 calves in a group on a beef-rearing unit that collected calves from farms in southern England (Stott et al., 1980). Calves were aged 32—52 days when first bled (time zero); they were bled again 6, 9, 15 and 21 weeks later.

Fifty sera were taken from a group of cattle in which some animals showed clinical mucosal disease, and 23 of which were shown to have a BVDV viraemia (Brownlie et al., 1984).

The standard serum used in the assay was prepared in a gnotobiotic calf by intranasal infection with live virus (strain IRAD C2415) followed by a subcutaneous injection of live virus (strain NADL) in Freund's complete adjuvant (Difco Laboratories).

To examine the specificity of the ELISA, antisera that had been prepared by hyperimmunisation of gnotobiotic calves with respiratory syncytial virus, parainfluenza virus type 3, *Mycoplasma dispar* and *Ureaplasma diversum*, and convalescent antisera from gnotobiotic calves infected with *Mycoplasma bovis* or infectious bovine rhinotracheitis virus were tested.

Fractionation of bovine sera

Three bovine sera were examined to determine whether virus neutralizing activity was associated with IgM, IgGl or IgG2 antibody. One of these sera was from the gnotobiotic calf immunised with BVDV (Sample A, Table I), the other 2 were adult cow sera. The IgM, IgGl and IgG2 fractions were prepared by chromatography and tested by double diffusion (Fey et al., 1976).

TABLE I

Antibody to BVDV in purified Ig fractions

Sample[a]	Titre by ELISA with antibody to:			Titre by neutralization
	IgG1	IgG2	IgM	
A. Serum	5000	200	< 50	3072
IgG1	2400	350	< 10	1024
IgG2	< 10	< 10	< 10	⩽ 3
IgM	< 10	< 10	< 10	⩽ 3
B. Serum	4000	400	50	2048
IgG1	3000	30	< 10	1024
IgG2	< 10	350	< 10	512
IgM	20	10	10	⩽ 3
C. Serum	6000	800	< 50	8192
IgG1	3000	10	10	768
IgG2	< 10	350	< 10	32
IgM	20	< 10	< 10	8

[a]Serum and immunoglobulin fractions.

RESULTS

Optimum conditions for the test

Preliminary checkerboard titrations established the optimum concentration of antigen, method of sensitising and choice of microtitre plates. Addition of antigen in PBSa or carbonate buffer pH 9.6, and overnight incubation without drying were not successful in adsorbing BVDV antigens onto plates. Rigid polystyrene plates were not compatible with the methods employed here, when antigens were added in either of the 2 buffers described or when the antigens dried on. Optimum concentrations of anti-Ig were determined in the usual manner. Nonidet extracted antigens could be stored at both −70° C and 4° C for at least 6 months; the longest times examined. Sensitised dried plates could also be stored sealed with Sellotape for 6 months at 4° C.

Comparison of the single dilution assay and the end-point titration method

In order to determine whether values obtained by the single dilution assay were quantitative, a comparison was made between specific IgG1 and IgG2 antibody titres, determined by a single 1/100 dilution of unknown sera with units of antibody read from a standard curve, or by the end-point titration method. Twenty-two sera were examined. These included 10 taken from calves 4.5 weeks after experimental infection with BVDV (Nuttall et al., 1980), 10 sera from normal adult cows, and 2 sera from cattle with BVDV viraemia.

For IgG1 antibody, comparison of end-point titres and number of units of antibody gave a correlation coefficient (r) = 0.882 ($P < 0.001$): for IgG2 antibody r = 0.949 ($P < 0.001$). Thus, it was established that the single dilution method was a satisfactory quantitative system in the assay as performed here, and it was used subsequently.

Comparison of ELISA with virus neutralization tests

Ninety-five samples were examined for antibody to BVDV by the ELISA and the virus neutralization test. These samples included the 10 pairs of sera taken from calves before and 4.5 weeks after intranasal infection with BVDV, sera from 41 adult cows, colostrum from 13 cows and sera from 21 calves taken after the feeding of colostrum. The ELISA was performed using anti-bovine IgG1, or IgG2, or IgG1 + IgG2.

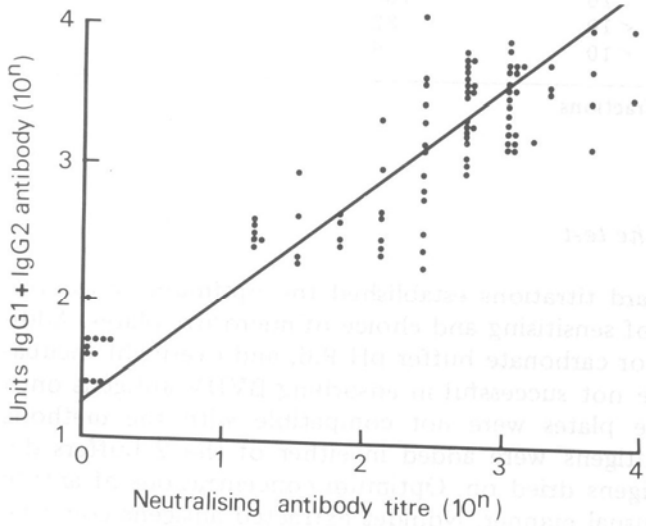

Fig. 1. A comparison of ELISA and the neutralization assay for measuring antibody to BVDV. The method assayed IgG1 and IgG2 antibodies together. Ninety-five samples were compared.

The correlation coefficient (r) for units of IgG1 antibody and neutralization titres was 0.86 ($P < 0.001$): for IgG2 antibody $r = 0.007$ ($P > 0.1$); for IgG1 + IgG2 antibody $r = 0.89$ ($P < 0.001$) (Fig. 1).

In a further study, the IgM, IgG1 and IgG2 fractions were prepared from 3 sera, and the virus neutralization titres and specific IgM, IgG1 and IgG2 antibody titres of those fractions were determined by the end-point titration method (Table I). Both IgG1 and IgG2 fractions were detected by ELISA and neutralization, but no BVDV antibody was convincingly demonstrated in the IgM fractions.

Specificity of ELISA

The hyperimmune or convalescent sera prepared in gnotobiotic calves against parainfluenza type 3 virus, respiratory syncytial virus, infectious bovine rhinotracheitis virus, *Mycoplasma bovis*, *M. dispar* and *Ureaplasma diversum*, all contained < 50 units of IgG1 or IgG2 antibody to BVDV per ml. Thus, the assay appeared specific.

Comparison of isotype response in cattle by end-point titration

To establish the predominant isotypes of bovine antibody to BVDV the specific IgG1, IgG2, IgM and IgA antibody titres were measured in a group of 9 sera from adult cows from the Compton herd and in 10 pairs of sera taken from calves before and after infection with BVDV.

In all the calf sera, pre-inoculation titres of IgG1, IgG2, IgM and IgA antibodies were ⩽ 25. All 10 calves had IgG1 antibodies detectable in post-inoculation sera. The titres ranged from 100 to 2700 (geometric mean titre ± standard deviation [SD] = $10^{2.70 \pm 0.529}$) six of the 10 calves produced an IgG2 antibody response. The titres ranged from 50 to 2400 (geometric mean titre ± SD = $10^{1.93 \pm 0.646}$). Only 2 animals had detectable IgM antibodies, the titres being 50, pre-inoculation titres were < 25 in these calves. None of the calves had detectable IgA antibodies.

All 9 sera from adult cows contained IgG1 and IgG2 antibodies. No IgM or IgA antibodies were detected in any of the sera. The IgG1 antibody titres ranged from 150 to 1100 (geometric mean titre ± SD = $10^{2.79 \pm 0.339}$) and the IgG2 antibody titres ranged from 65 to 3400 (geometric mean titre ± SD = $10^{2.75 \pm 0.568}$).

It was concluded that assays should determine IgG1 and IgG2 antibody, but determination of IgM and IgA was of no extra advantage.

IgG1 and IgG2 antibodies to BVDV in cattle sera

The mean number of units of specific IgG1 and IgG2 antibody to BVDV in the sera of 41 cows, colostrum from 13 of these cows, and sera from 21 calves taken after ingestion of colostrum are shown in Table II. Also given

are levels of specific IgG1 and IgG2 antibody to BVDV in 10 pairs of sera taken before, and 4.5 weeks after intranasal infection with BVDV. Only one of the 41 cows did not contain detectable antibody. Both IgG1 and IgG2 antibody were present in the remainder. Antibody to BVDV in colostrum and in sera from calves that had ingested colostrum was predominantly IgG1. Before exposure to BVDV all 10 experimentally-infected calves contained $\leqslant$ 50 units IgG1 antibody per ml of serum; the serum from one calf contained 100 units of IgG2 antibody while the other 9 sera contained $\leqslant$ 50 units IgG2 antibody. After injection both IgG1 and IgG2 antibodies to BVDV were detected in these calf sera.

TABLE II

Comparison of levels of IgG1 and IgG2 antibody to BVDV in sera and colostrum

Sample group	No. of animals	Geometric mean No. of units of antibody				Ratio IgG1 : IgG2
		IgG1	± SD	IgG2	± SD	
Adult cows	41	$10^{3.20}$	$\pm^{0.559}$	$10^{2.9}$	$\pm^{0.482}$	1.8 : 1
Colostrum	13	$10^{3.52}$	$\pm^{0.310}$	$10^{2.14}$	$\pm^{0.539}$	24 : 1
Colostrum-fed-calves	21	$10^{3.33}$	$\pm^{0.407}$	$10^{2.10}$	$\pm^{0.371}$	17 : 1
Post-inoculation calf sera[a]	10	$10^{2.24}$	$\pm^{0.238}$	$10^{1.93}$	$\pm^{0.413}$	2 : 1

[a]Pre-inoculation of calf sera contained $\leqslant 10^{1.7}$ units, IgG1 and IgG2 antibody except for one calf with 10^2 units IgG2.

Appearance of antibody to BVDV in sera from calves aged up to 8 months

Our results indicated that in order to maximise the chance of detecting antibody to BVDV, IgG1 or IgG2 antibody should be assayed or a system designed that detected both.

To examine this further, sera collected over a 24-week period from 44 calves, held together on a beef-rearing farm, were assayed. Calves were first bled at 5—7.5 weeks of age and 3, 6, 15 and 24 weeks later. The cumulative number of calves with a $\geqslant$2-fold increase in the number of units of IgG1, IgG2 or IgG1 + IgG2 antibody during the period studied was determined. The combined system that measured IgG1 + IgG2 antibody appeared marginally superior, particularly when maternal IgG1 was present; it detected an antibody rise to BVDV in 2, 8, 26 and 36 animals, with 2, 3, 27 and 36 rises detected using anti-IgG1, or 2, 4, 18 and 34 using anti-IgG2. Both the combined system and anti-IgG1 system detected 36 of 44 (82%) of calves in this group which had produced antibody responses to BVDV by the time they were 29—31.5 weeks old.

Antibody in viraemic cattle

Fifty sera were examined from a herd with mucosal disease, 23 of which were shown later to have persistent BVDV viraemias. Antibody was measured in sera from these animals using the combined anti-IgG1 + IgG2 system. Of the 23 viraemic cattle, 20 had $\leqslant 50$ units of antibody per ml of serum, the remaining 3 sera had $10^{2.0}$, $10^{2.2}$ and $10^{2.4}$ units of antibody per ml. The range of values for the normal non-viraemic animals in the same herd was $10^{2.2}$—$10^{4.1}$ units; geometric mean ± SD = $10^{3.4 \pm 0.40}$.

DISCUSSION

The end-point titration method has been recommended as the standard assay against which other systems can be compared (De Savigny and Voller, 1980). Although variations in antibody affinity as well as titre are known to affect values obtained in a single-dilution assay (Butler et al., 1978), we have shown that this test was quantitative and gave values comparable to the end-point titration method. On the basis of these results we used the single dilution assay in subsequent tests.

Initial experiments to establish which Ig isotypes were produced following BVDV infection were necessary because of the restricted isotype response sometimes observed following infection. Examination of sera from experimentally- and naturally-infected cattle indicated antibody was predominantly IgG1 and IgG2. Low levels of IgM antibody may not have been detected as a result of competitive inhibition between IgM and IgG antibodies (Chantler and Diment, 1981). However, the results obtained with fractionated sera do not substantiate this view. These studies indicated both IgG1 and IgG2 antibody had neutralizing activity for BVDV and also that the IgM fraction did not contain a substantial proportion of the neutralizing antibody in bovine sera. The neutralizing activity of the IgM fraction of Sample C could have been due to the presence of a small amount of IgG1 antibody in this sample. The high and prolonged IgM response suggested by Fernelius (1966) based on comparisons of neutralization and precipitation tests in fractionated sera, was not observed. Subsequent studies therefore concentrated on tests designed to assay only the two IgG subclasses.

The ratio of IgG1 : IgG2 antibody was about 2 : 1 in sera from naturally- and experimentally-infected cattle. In colostrum and sera from colostrum-fed calves the ratio of IgG1 : IgG2 antibody was about 10 times higher. This is as expected from established data on the selective transfer of IgG1 into colostrum (Butler, 1983). It is also the obvious explanation for the poor correlation between IgG2 antibody measured by ELISA and the neutralization assay, despite the demonstrated ability of IgG2 fractions of sera to neutralize BVDV.

In the assays, control wells were sensitised with Nonidet extracted non-infected calf-testis cells. This enabled non-specific binding of sera to be

eliminated, although it may have resulted in some sera that contained low levels of antibody and that produced high non-specific binding being recorded as giving a negative reaction. Pre-inoculation sera from conventionally-reared calves held in isolation that were devoid of serum antibody by the neutralization test contained no antibody detected by ELISA. Also, the antisera prepared in gnotobiotic calves to other agents did not react in the test indicating that non-specificity was not a problem.

It is concluded that, optimally, assays should detect both IgG1 and IgG2 antibody although an assay that detected just IgG1 would be nearly as effective in diagnosing infection and measuring maternal antibody. The viraemic cows had no detectable, or very low amounts of, antibody to BVDV and the assay should provide a rapid method for detecting immunologically-compromised cattle at risk of developing clinical mucosal disease. It should also be useful for following the immune response to BVDV and detecting infections with this virus. The high proportion of adult cattle with antibody (40/41), and the detection of antibody responses in calves by the time they were 8 months old (36/44) is consistent with other data, and indicates the high risk of infection and high prevalence of BVDV in such cattle.

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