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## Seroepidemiology of Breda Virus in Cattle using ELISA

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### ABSTRACT

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Two direct blocking enzyme linked immunosorbent assays (ELISA) for the detection of antibodies to Breda virus in sera of cattle were compared. An ELISA with consecutive addition of antigen and test serum to an antibody-coated plate gave higher positive: negative absorbance ratios than an ELISA in which antigen and test serum were added simultaneously.

Sera collected from breeding and fattening herds in The Netherlands ( $n = 1313$ ) and the F.R.G. ( $n = 716$ ) were tested, and antibodies to Breda virus were demonstrated in 94% of adult cattle. Ninety percent of newborn calves had high levels of maternal antibodies, which waned until the age of 3 months. Active seroconversion occurred between 7 and 24 months in most animals.

### INTRODUCTION

During an outbreak in 1979 of neonatal calf diarrhea in a beef herd in Breda, IA, U.S.A., a new virus was detected. This "Breda virus" (BRV) is morphologically and antigenically different from known bovine viruses and causes diarrhea in gnotobiotic and colostrum-deprived calves (Woode et al., 1982). An antigenic relationship of BRV with Berne virus (BEV) was discovered (Weiss et al., 1983); BEV has been extensively characterized (Weiss and Horzinek, 1987; Horzinek et al., 1987a,b) and proposed as prototype of the new family Toroviridae (Horzinek and Weiss, 1984; Horzinek et al., 1987a,b). Other Breda viruses were found in the feces from a colostrum-deprived calf in Ames, IA (Woode et al., 1983) and in 5-6-month-old diarrheic calves in Ohio (Saif et al., 1981). All viruses shared antigens as determined by enzyme-linked im-

munosorbent assay (ELISA), with 6–10-fold differences between the homologous and heterologous reactions. For this reason and on the basis of hemagglutination inhibition tests and immune electron microscopy the three isolates were assigned to two serotypes: Breda virus Serotype 1 (BRV1) is represented by the original Breda, IA isolate, and Serotype 2 (BRV2) comprises the Ohio isolate and the second Iowa isolate (Woode et al., 1985).

So far, all attempts to grow BRV *in vitro* have failed (Woode et al., 1982); recent observations by Lamouliatte et al. (1987) need to be confirmed. For this reason, only limited studies on the characterization of the virus (Koopmans et al., 1986), its diagnosis, pathology and epidemiology (Woode, 1987) were done. In neonatal calves, BRV infections spread rapidly with diarrhea commencing as early as 1–3 days after birth (Woode, 1987). Large amounts of virus ( $10^7$  viral hemagglutinating units  $\text{ml}^{-1}$ ) were detectable in feces 3–4 days after infection. In experimentally infected calves incubation periods ranged from 20 to 172 h (Woode et al., 1982). The role of older cattle and of other animal species in BRV epidemiology is not known.

In the present communication we describe the distribution of BRV antibodies in The Netherlands and the F.R.G. and some epidemiological aspects of the infection; the data were obtained using two modifications of the ELISA method.

## MATERIALS AND METHODS

### *Preparation of antigen*

We chose to use BRV2 in our studies because it is relatively stable as compared with BRV1 (Koopmans et al., 1986). A gnotobiotic calf (GC68) was fed a suspension of BRV2 (Iowa isolate at Passage 3 in GC) at 1 day of age; diarrheic fecal samples were collected at 48 and 72 h. No other virus was observed to be present, by electron microscopy, hemagglutination and tissue-culture methods, including bovine coronavirus, rotavirus, astrovirus, parvovirus and bovine pestivirus (BVDV). A 20% suspension in phosphate buffered saline (PBS) was clarified by low speed sedimentation ( $3000 \times g$  for 20 min) and virus from the supernatant was spun through a 15% (w/w) sucrose solution onto a cushion of 50% (w/w) sucrose. The interphase was collected, diluted 3-fold in PBS, layered on top of a linear 10–50% sucrose gradient and centrifuged to equilibrium at  $50\,000 \times g$  for 16 h. The peak fractions ( $1.17 \text{ g ml}^{-1}$ ) were used as antigen in ELISA.

### *Preparation of capture antibody and conjugate*

A gnotobiotic calf (GC76) was fed BRV2 (Iowa isolate at Passage 3 in GC) at 1 day of age. Breda virus was extracted from the diarrheic feces of this calf and semipurified by ultracentrifugation through 20% sucrose. At 26 and 40

days post-infection, the calf was vaccinated intramuscularly with BRV2 with Freund's incomplete adjuvant and at 54 days post-infection with BRV2 without adjuvant. The calf was bled 12 days later. The hyperimmune serum was shown to be free of antibodies to rotavirus, bovine coronavirus, astrovirus and bovine pestivirus (BVDV) and by electron microscopy and tissue-culture isolation methods, no other virus was observed to be present in the feces. Immunoglobulins were precipitated from the serum by salt fractionation (Hudson and Hay, 1980) and quantified using the Lowry method.

A fraction of the purified IgG was conjugated with horseradish peroxidase as described by Nakane and Pierce (1967).

### *Origin of the sera*

For the serosurvey, bovine sera were obtained from Veterinary Diagnostic Institutes in Arnsberg ( $n=100$ ), Nürnberg ( $n=140$ ) and Neumünster ( $n=151$ ) and from the Virology Department of the Veterinary School in Hannover, F.R.G. ( $n=100$ ); additional samples were collected at farms in Hessen and Baden-Württemberg, F.R.G. ( $n=225$ ). The sera representing the Dutch cattle population were obtained from Animal Health Services in Overijssel ( $n=617$ ), Gelderland ( $n=222$ ), Friesland ( $n=48$ ) and West/Midden Nederland ( $n=78$ ), the Central Veterinary Institute (CVI), Lelystad ( $n=212$ ), and the Department of Large Animal Medicine of the Veterinary Faculty in Utrecht ( $n=136$ ). The sera obtained from the Dutch Animal Health Services and the German Veterinary Diagnostic Institutes were collected for regular screening purposes. The CVI sera included 16 sera from specified pathogen-free (SPF) calves and paired samples from milking cows from farms where a tentative diagnosis of winter dysentery was made, based on the following signs: outbreak of rapidly spreading scours in milking herds with a morbidity rate approaching 100%, lasting 3–4 days and resulting in a sharp drop in milk production (Hoyer et al., 1985). The sera from the Veterinary Faculty in Utrecht included 10 paired samples from an outbreak of acute respiratory disease in 3-month-old calves and six sera from newborn calves with blood protein levels below  $40 \text{ g l}^{-1}$  as a result of insufficient colostrum intake. The sera were coded and stored at  $-20^\circ\text{C}$  until testing was done.

In addition, 10 colostrum samples from healthy dairy cows were tested.

### *Principle of the assay*

A direct blocking ELISA was used for mass serology. Advantages of this test are that antibodies of different Ig classes and from different animal species can be detected, and that antibodies are detected also at low concentrations (Eilens, 1981).

A modification of the method described by Brown et al. (1987) was em-

ployed. In short, the wells in a polystyrene plate were coated with purified bovine anti-BRV2 immunoglobulin (capture antibody, BaBRV2Ig) diluted 1:1500 in 0.1 M  $\text{Na}_2\text{CO}_3/\text{NaHCO}_3$  pH 9.6 by overnight incubation at 37°C; all subsequent incubation steps were done in 100- $\mu\text{l}$  volumes at 37°C for 1 h unless indicated otherwise. To remove excess immunoglobulin, the plates were washed with a solution of 0.15 M NaCl containing 0.5% Tween 80. From this point onward, two different techniques were followed.

In the first approach (consecutive method) a 1:400 dilution of the BRV2 antigen preparation, purified from calf feces was added to the coated wells in ELISA buffer 1 (PBS supplemented with 0.35 M NaCl, 1 mM EDTA pH 7.5 and 0.05% Tween 80). The plates were washed and the field serum to be tested was added at a 1:40 dilution in ELISA buffer 2 (PBS supplemented with 0.65 M NaCl, 1 mM EDTA pH 7.0 and 0.05% Tween 80). After another rinse, purified bovine anti-BRV2 immunoglobulin conjugated to horseradish peroxidase (BaBRV2Ig-PO) diluted 1:250 in buffer 2 was added, and bound conjugate was visualized after adding the substrate (0.05 M citric acid, pH 4, containing 0.008%  $\text{H}_2\text{O}_2$  and 0.2 mM 2,2'-azinodi-(3-ethylbenzthiazoline sulfonic acid). High absorbance is seen in the absence of anti-BRV antibodies whereas the reaction is blocked when the serum contains antibodies. Hyperimmune sera against BRV2 and cryptosporidia or rotavirus, prepared similarly to BaBRV2Ig, served as positive (100% blocking) and negative (0% blocking) reference preparations. All the sera from The Netherlands were tested in this system.

In the other type of test (simultaneous method), antigen and test serum were mixed in a separate microtiter plate. After 1 h of incubation, the mixture was pipetted into the BaBRV2Ig-coated plates. The remaining steps were similar to the consecutive test method. All sera from Germany were tested in this way.

A comparison was made between both methods by testing 84 serum samples in parallel. The blocking results were grouped into one of the following clusters: 0–20%, 20–50%,  $\geq 50\%$  inhibition of binding of BaBRV2Ig-PO.

Positive to negative (P/N) ratios were determined for both ELISA modifications by testing a known positive (GC76) and negative (GC75) serum 30 times in parallel.

#### *Optimisation of the assay*

The optimal combination of BaBRV2Ig concentration and BRV2 dilution was determined by checkerboard titration: a microtiter plate was coated with serial 2-fold dilutions of BaBRV2Ig in coating buffer and serial 2-fold dilutions of the antigen in ELISA buffer 1 were added. We used a combination of dilutions giving P/N ratios of  $\geq 3$  in our routine testing.

The optimal conjugate concentration was determined by testing serial 2-fold

dilutions in ELISA buffer 2 at constant antigen and Ig-coating concentrations. A dilution with a P/N ratio of  $\geq 3$  was used in all subsequent experiments.

The results are expressed as blocking percentages; they were calculated using the formula:

$$\text{Percentage blocking} = \frac{(A_{\text{neg}} - A_t)}{(A_{\text{neg}} - A_{\text{pos}})} \times 100$$

$A_t$  = mean absorbance at 405 nm of the test serum;

$A_{\text{neg}}$  = mean absorbance of the negative reference serum;

$A_{\text{pos}}$  = mean absorbance of the positive reference serum.

To detect a possible correlation between percentage of blocking and antibody titer the sera were assigned to 10 groups according to the percentage of blocking (0–10%, 10–20%, etc.) at a dilution of 1:40. From each group, 10 sera were selected and titrated in serial 3-fold dilutions in the consecutive ELISA. Titers were calculated from the highest serum dilution resulting in  $\geq 50\%$  blocking when compared with the positive and negative reference sera. The correlation coefficient was calculated using Pearson's test (Dixon and Massey, 1969).

## RESULTS

When using the consecutive method more animals were found negative for antibodies to BRV2 or had low blocking percentages (Table 1). A higher P/N ratio was also noted between positive and negative reference sera, when tested in the consecutive ELISA method ( $4.431 \pm 0.426$ ) as compared with the simultaneous method ( $4.021 \pm 0.513$ ); this difference is significant ( $P < 0.001$ ,  $n = 30$ , Student's *t*-test).

The correlation coefficient between the blocking percentages and the serum titers in the consecutive ELISA was 0.86 ( $P < 0.0001$ , Pearson's test; Fig. 1).

As can be seen from Fig. 2, the sera with blocking percentages of  $< 10$  and  $> 90$  do not follow the normal distribution pattern. Expressed in serum titers,

TABLE 1

Percentage of sera per blocking group in the consecutive and the simultaneous ELISA ( $n = 84$ ). Sera were tested at a 1:40 dilution

Test	Blocking results		
	0–20%	20–50%	50–100%
Consecutive ELISA	31.0	19.0	50.0
Simultaneous ELISA	22.6	9.5	67.9

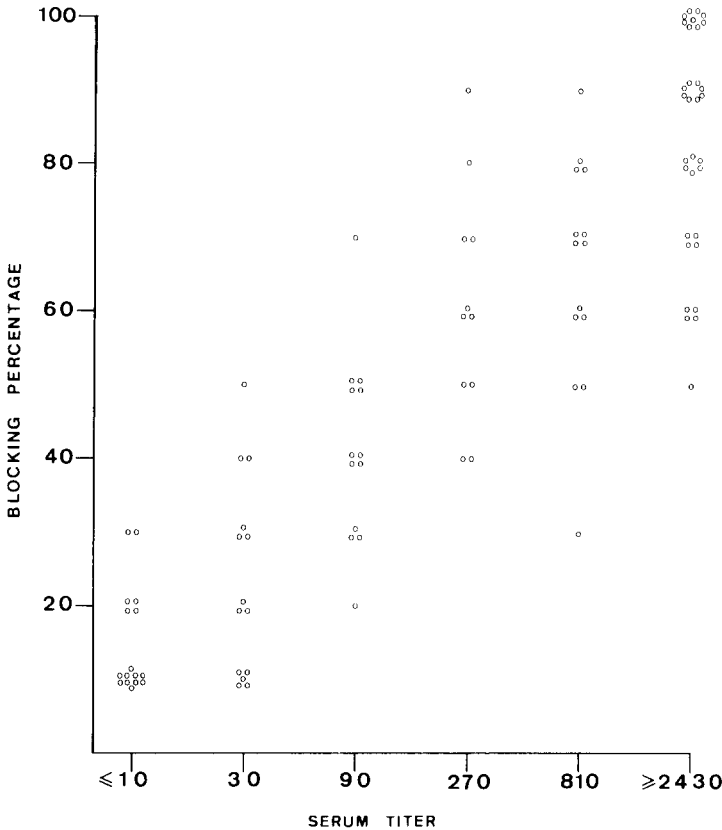


Fig. 1. Correlation between serum titer and blocking percentage at a serum dilution 1:40 ( $n=100$ ).

18.1% of our collection of sera from Dutch cattle ( $n=1313$ ) had values of  $\leq 30$  (0–20% blocking), 23.7% showed intermediate titers (20–50% blocking) of 30–90 and 58.2% had titers of 270–2430 and higher ( $\geq 50\%$ ).

Distributions of blocking percentages in sera from the F.R.G. and The Netherlands are shown in Fig. 2A and B; since most sera came from the Dutch provinces Gelderland and Overijssel we show the results obtained with these samples separately (Fig. 2C and D). When disregarding the 90–100% columns, a normal distribution can be recognized; median values are situated at 70–80% blocking in Fig. 2B and C (The Netherlands and Gelderland), and at 50–60% in Fig. 2A and D (Germany and Overijssel).

In Fig. 3, the percentages of animals with antibodies to BRV ( $\geq 20\%$  blocking, corresponding to a serum titer of  $\geq 30$ ) are shown for different age groups ( $n=244$ ). Ninety percent of calves  $< 1$  month old had antibodies, 98% at 2 months of age; then a gradual decline was noted until at approximately 14–16 weeks of age most calves were seronegative. When the animals had reached

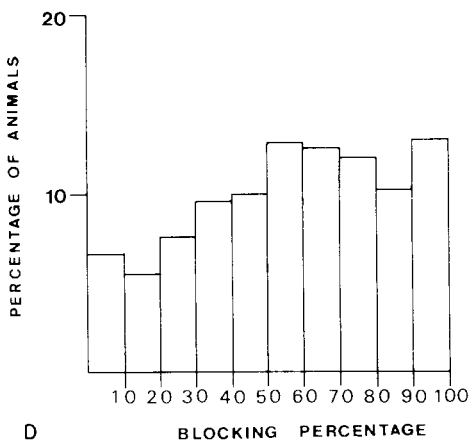
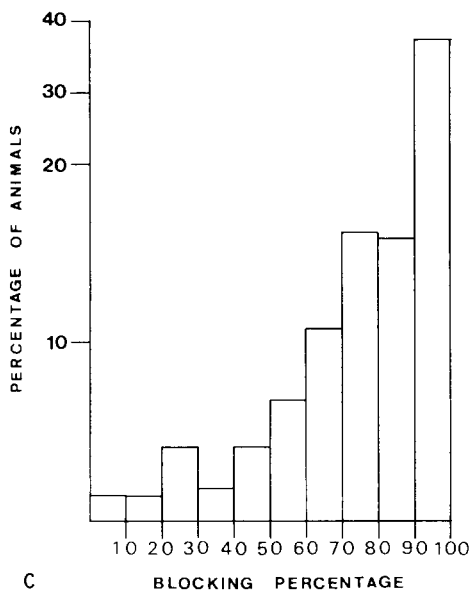
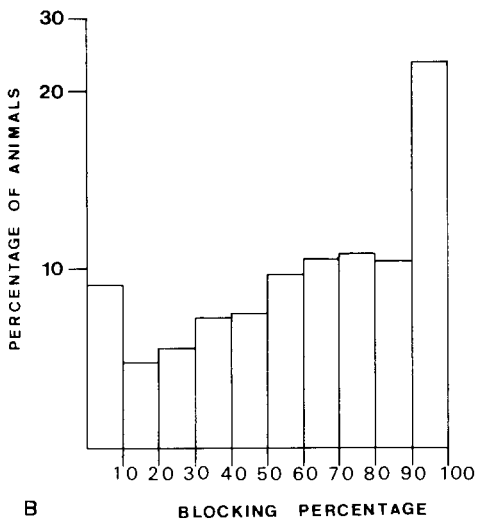
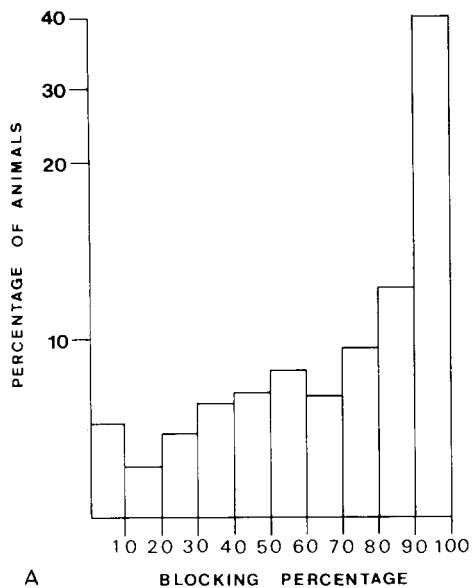


Fig. 2. Percentage of animals per blocking group in the F.R.G. (A,  $n=716$ , tested using the simultaneous method), The Netherlands (B,  $n=1313$ ), Dutch Provinces Gelderland (C,  $n=222$ ) and Overijssel (D,  $n=617$ ).



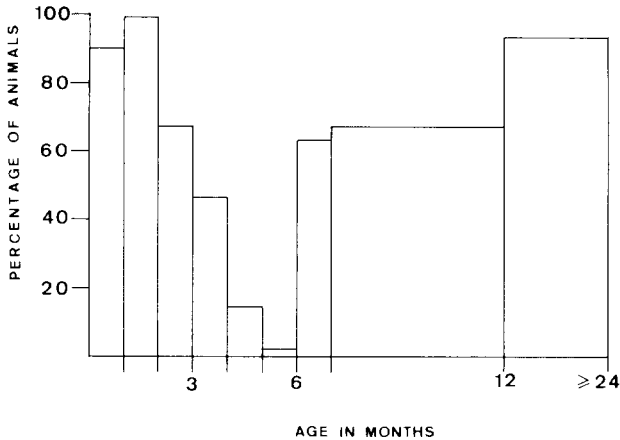


Fig. 3. Age distribution of antibodies to BRV2 in cattle. Seropositive animals are given as percentages of the sample ( $n=244$ ).

the age of about 6 months, seroconversion took place. From this age onward an increasing fraction of animals had antibodies to BRV, with 94.6% of adult animals detected in our populations; 61.3% of adult animals had blocking percentages of  $\geq 50\%$ , corresponding to titers of  $\geq 270$ . Six sera from adult bulls of a closed artificial insemination center as well as 16 sera from SPF calves and six sera of hypoproteinemic newborn calves had blocking percentages of  $\leq 15\%$ . All 10 colostrum samples had high levels of BRV antibodies (90–100% blocking).

In view of the regular occurrence of winter dysentery in The Netherlands, we were interested to know whether seroconversion to Breda virus antigens would occur. Paired serum samples from adult milking cows were, therefore, collected during outbreaks and were tested. Seroconversion was defined as  $> 25\%$  rise in percentage blocking between sera collected as soon as possible after the onset of disease, as compared with sera collected 2–3 weeks later. Antibody rises occurred in 4 out of 5 farms, with 40% ( $n=10$ ), 67% ( $n=9$ ), 47% ( $n=7$ ) and 29% ( $n=7$ ) seroconversion (average 45%).

In another group of paired sera, from 3-month-old calves with a history of acute respiratory disease, seroconversion was seen in four out of 10 animals. Three of these animals had low starting titers ( $\leq 30\%$  blocking).

## DISCUSSION

In our hands, the simultaneous ELISA resulted in comparatively more animals being grouped into the high blocking percentage columns. Partially antibody-covered BRV2 antigen (as should be found after preincubation in the simultaneous ELISA) will probably not bind as efficiently to the antibody-

coated polystyrene plate as BRV2 alone does. During the subsequent washing cycles these complexes may be rinsed away and no binding sites for the conjugate will be left, resulting in a false-positive blocking reaction. In combination with the higher P/N ratios determined for the consecutive ELISA we decided to use this method in all further experiments. At this point all German sera had already been assayed in the simultaneous test.

When the antibody concentration in a test serum is at equilibrium with the antigen, 100% blocking will occur. At higher antibody concentrations, however, the ELISA results will be the same, leading to an accumulation of animals in the 90–100% blocking group. This is likely to occur when tests are performed at a constant serum dilution (1:40 in our case). When disregarding these groups in the frequency distribution of blocking percentages, a normal distribution can be recognized. The graph for the sera from Germany has a maximum at lower values as compared with the graph from The Netherlands. The different test system used for the German sera does not explain this lower median value: a higher percentage of seropositive animals should be expected, as discussed above, leading to a right shift of the distribution of blocking percentages. A similar difference was observed within the Dutch sera between the Provinces Gelderland and Overijssel. One reason for this finding may be a lower incidence of BRV infections in farms in Overijssel and Germany, resulting in less frequent booster infections. A second possibility is that different sampling strategies between the Animal Health Services would give similar results: if a relatively high percentage of animals between 3 and 6 months of age is tested, when most calves are seronegative, lower overall values may be calculated; the age was unknown for many animals. Third, a torovirus serotype, different from that in our test, may exist in cattle in Europe. When studying our ELISA results per animal, lower average blocking percentages are found in the Overijssel area; therefore, the presence of a different serotype in this province is a possibility.

Ninety percent of newborn calves had antibodies to BRV. Antibodies were absent in specified pathogen-free calves from the Central Veterinary Institute, Lelystad, and in 1-day-old diarrheic calves from the Department of Large Animal Medicine with low blood protein levels as a result of insufficient colostrum intake. This indicates that the BRV antibodies in young calves are maternally derived as has been found for antibodies to other pathogens (Snodgrass and Wells, 1978); indeed all colostrum samples tested had high levels of antibodies to BRV. The rise in percentage of seropositive animals at 2 months of age indicates an infection in the 1-month age group. For rotaviruses, enteric infection in the presence of circulating antibodies has been proven (Moerman et al., 1982; Snodgrass and Wells, 1978). We have preliminary data indicating that a similar situation exists for BRV. The sudden seroconversion of most calves at the age of 6–7 months coincides with the time they are stabled after the grazing period; this leads to closer contact with older cattle and exposure

to infection. Crouch and Acres (1984) have shown that up to 44% of adult cows excrete rotavirus and 77% coronavirus with their feces. The role of fecal spread from seropositive cows in BRV epidemiology remains to be studied.

Our findings of 94.6% positive serum samples are in agreement with those of other authors reporting 88.5% by ELISA in the U.S.A. (Woode et al., 1985) and 86% by neutralization test in Switzerland (Weiss et al., 1984); in Gt. Britain, 55% of cattle was found seropositive (Brown et al., 1987). In that study, however, a serum was considered to contain BRV-specific antibodies at > 60% blocking. When using the same threshold in the interpretation of our results a comparable 56% of Dutch cattle ( $n=1313$ ) would be found positive. The higher percentages, however, are more likely to reflect the true distribution, since they have also been found in neutralization tests with their intrinsically higher sensitivity.

Both BRV serotypes have been isolated from outbreaks of diarrhea and are pathogenic under experimental conditions (Woode, 1987). The pathogenicity under farm conditions, however, remains to be proven. With this intention we have tested paired serum samples from field outbreaks of winter dysentery. In four out of the five tested farms, a varying percentage of animals (29–67%) showed a distinct antibody rise at the time of the second testing. The seroconversion seen in cattle aged 7–24 months (Fig. 3) does not account for this high number of seroconversions, since only milking cows were tested. Further studies are needed to examine the role of BRV in this disease. Serological evidence for infection with bovine coronavirus in outbreaks of winter dysentery in Japan has been presented (Takahashi et al., 1983); in The Netherlands no such evidence was found (P.W. de Leeuw, personal communication, 1987).

The seroconversions seen in sera from four out of 10 calves, aged 3 months and with a history of acute respiratory disease, may be coincidental although in experimental infections with BRV1, ocular discharge and hyperpnea were seen (Woode et al., 1982).

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