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Résumé
DETECTION DES ANTICORPS SPECIFIQUES DU ROTAVIRUS DANS LE COLOSTRUM ET LE LAIT PAR LA TECHNIQUE ELISA. — Cet article décrit la technique ELISA pour la détection et la titration des anticorps spécifiques du rotavirus dans le colostrum. Les résultats obtenus étaient en corrélation positive avec ceux d’un test de neutralisation des anticorps.

Des colostrum furent obtenus d’une même ferme pendant une période de 18 mois. Aucune relation n’a été observée entre le titre colostral en anticorps juste après la mise bas et l’apparition de diarrhées dues au rotavirus chez les veaux. Dans une seconde ferme, seuls les échantillons obtenus pendant la saison de vêlage ont été dosés. Au cours de cette courte période, les titres élevés d’anticorps colostraux semblaient réduire l’incidence de la diarrhée parmi les veaux et différer l’apparition de l’excrétion de rotavirus dans les fèces. Ces résultats sont discutés en relation avec la décroissance rapide du contenu en anticorps du colostrum après le vêlage.

Introduction
Rotavirus-specific immunoglobulins or colostrum containing specific antibody, are capable of preventing rotavirus induced diarrhoea in calves and lambs, if administered orally a few hours before or after challenge by the same route (Bridger and Woode, 1975; Snodgrass and Wells, 1976). However, once absorbed from the gut, specific antibody does not appear to be protective (Mebus et al., 1973; Snodgrass and Wells, 1976). Thus, protection of new-born calves and lambs against rotavirus induced diarrhoea probably depends on the continuous presence of sufficient amounts of specific antibody in the alimentary tract. Whether a relationship exists between the initial colostrum titer and the occurrence and time of appearance of diarrhoea in bucket reared calves in the field, has, to our knowledge, not been reported. Yet such data are important when considering the possibility of vaccination of dairy cows, or the prolonged use of first-day colostrum (Woode et al., 1975). In a study along these lines we had to examine large numbers of colostrum samples. Neutralizing antibody assay was found to be too cumbersome and time consuming. Therefore the usefulness of the enzyme-linked immunosorbent assay (ELISA) for the titration of rotavirus specific antibody in colostrum was examined.
In this report the techniques used are described and some preliminary results are presented.

Materials and methods

Collection of field materials and clinical observations

Investigations were carried out on two dairy farms into the prevalence of neonatal calf diarrhoea and its relationship with rotavirus excretion in the faeces. All female calves were observed daily the first three weeks after birth. On one farm the survey ranged from January 1976 to June 1977 and on the other farm from February to May 1977. On the first farm all calves were fed twice daily with 2.5 liters of colostrum of their own dam during the first three days; thereafter the same amount of pooled milk was given. After the tenth day milk replacer was introduced into the diet. On the second farm each calf received colostrum ad libitum by means of a continuously filled nipple-bucket during the first four days. From the fifth to the eighth day they were fed two liters of milk twice daily and thereafter milk replacer.

Colostrum samples were obtained the first time the cows were milked, usually immediately after calving, and stored at —20°C. Two faecal samples were obtained from the calves during the first five days of life, and additional samples were taken when diarrhoea developed later. All samples were stored at —20°C. They were examined for the presence of rotavirus either by electron microscopy (de Leeuw et al., 1977) or by enzyme-linked immunosorbent assay (Ellens and de Leeuw, 1977).

ELISA

The ELISA for the titration of rotavirus-specific antibody in colostrum was essentially carried out as the blocking assay used to test the specificity of the rotavirus-ELISA (Ellens et al., 1978).

Polystyrene microtiter plates (Cooke) were coated with 100 µl of the globulin fraction of a bovine anti-rotavirus serum diluted 1:2500 in 0.05 M carbonate buffer pH 9.6. After an incubation period of 18 hours at 37°C the plates were rinsed three times with tap water containing 0.05% Tween 80, and subsequently incubated for three hours at 37°C with 100 µl of a 1:100 dilution of a standard rotavirus positive faecal extract. The plates were then rinsed three times and incubated for one hour with 100 µl volumes of colostrum, twofold serially diluted from 1:100 to 1:6400. After another washing procedure 100 µl of a 1:2500 dilution of the conjugate, horseradish peroxidase coupled by glutaraldehyde to bovine anti-rotavirus immunoglobulin-G, was added. A further incubation period of one hour at 37°C, was followed by a threefold rinse and addition of 100 µl of the substrate solution, containing 1 mg per ml 5-aminosalicylic acid (recrystallized in the presence of sodium disulphite), 0.005% hydrogen peroxide in 0.01 M sodium phosphate and 0.1 mM Na2EDTA, pH 6.0 (Ellens and Gielkens, in preparation).

The plates were read by visual inspection 18 hours after the substrate had been added. A serially diluted standard sample was incorporated in each test and used as a reference. In some experiments the extinction at 474 nm of the contents of the wells was measured.

ELISA titers are expressed as the reciprocal of the colostrum dilution giving a colour corresponding with an extinction of 1.0.

Neutralizing antibody assay

Colostrum samples were threefold serially diluted in Hanks' BSS and mixed with an equal volume of a cell culture adapted rotavirus* suspension containing 200-400 TCID50 per ml. After 1 hour at 37°C, 0.5 ml volumes were inoculated onto secondary bovine embryo kidney cell monolayers. The cells were grown on coverslips in Leighton tubes; the growth medium used was Hanks' BSS with 10% foetal calf serum, 0.5% LAH and appropriate antibiotics. Two tubes per dilution were used. Before incubation the monolayers were washed with Hanks' BSS. During the adsorption period of 1 hour at 37°C all tubes were rolled. The monolayers were then washed again, maintenance medium (Eagle's without serum) was added, and the cultures were incubated in a roller drum at 37°C for 48 hours. After washing with PBS, the cultures were fixed

* Kindly provided by Dr. N. Zygraich, RIT, Rixensart, Belgium.
in acetone and stained with a 1:35 dilution of FITC conjugated rabbit anti-rotavirus serum. This was followed by washing in PBS before mounting in buffered glycerol and examination with an U.V. microscope. Neutralization test (NT) titers of colostrum are expressed as the reciprocal of the highest dilution preventing the formation of fluorescent cells.

Results

The extinction profiles of ELISA, obtained with two serially diluted colostrum samples, are given in fig. 1. Based on these and similar results the end-point was taken as the dilution giving an $E_{474}$ of 1.0. In this area the slope of the curve is about one extinction unit per twofold dilution, which permits visual reading.

The rotavirus antibody titer of 216 colostrum samples analysed with ELISA varied between <100 and 6400. The hyperimmune bovine anti-rotavirus serum, used as an internal standard in each test, had a mean titer of 2400. For comparison 35 colostrum samples were titrated by neutralizing antibody

![ELISA extinction values for serial dilutions of two colostrum samples.](image1)

![Correlation between ELISA and NT titers of 35 colostrum samples.](image2)

![ELISA titers of colostrum samples obtained twice daily after calving.](image3)
assay. An over-all positive correlation between the results of both tests was found (Fig. 2).

To examine the excretion of rotavirus-specific antibody in colostrum at different times after calving, colostrum samples were obtained from 15 cows the first six times they were milked. After approximately 24 hours ELISA titers had generally fallen to one quarter of the original value. Fig. 3 shows two representative examples. A similar rapid decline was also observed in the NT titers of these samples.

On both farms rotavirus-associated diarrhoea in calves was mainly observed between the fourth and the fourteenth day of life. A detailed account of these results will be published elsewhere (de Leeuw et al., in preparation). A total of 125 colostrum samples were obtained on the farm that was followed for 18 months. Titers of less than 100 were found in six samples. The mean colostral antibody ELISA titer was highest during the second and third quarters of 1976 and declined thereafter (Fig. 4). The percentage of calves developing rotavirus-associated diarrhoea (i.e. rotaviruses detected in the faeces somewhere near the beginning of the diarrhoea) was 60 to 70% during the first quarter of both 1976 and 1977. Virtually no calves developed diarrhoea during the summer months. This seasonal pattern of diarrhoea also appeared in 1977, although the mean ELISA titer during the second quarter of this year was considerably lower than in 1976 (Fig. 4). On an individual basis no relationship was found between the ELISA titer of colostrum and the occurrence of rotavirus-associated diarrhoea in calves.

Colostrum samples from 91 cows were obtained on the other farm, on which there was a distinct calving season from January to May. During the observation period the mean rotavirus antibody titer declined from 1500 ± 300 (n = 29) in February to 700 ± 100 (n = 27) in April. Rotavirus excretion of calves born in February occurred in general later than that of calves born in April: on day 9 ± 1 and 6 ± 1, respectively.

Colostrum fed to calves which did not develop diarrhoea had a significantly higher mean antibody titer than that fed to calves which developed rotavirus-associated diarrhoea (1500 ± 260 and 650 ± 100, p < 0.01).

Discussion

For the detection of antibody in colostrum the ELISA was carried out as a blocking test rather than using the indirect method (Voller et al., 1976). The latter requires the use of enzyme labeled anti-species immunoglobulins and purified rotavirus or heterologous anti-rotavirus gammaglobulins (Scherrer and Bernard, 1977; Yolken et al., 1978) for coating the plates. For a visual reading of the indirect assay the dilution factor has to be more than twofold which implicates that standard microtiter diluters cannot be used. On the other hand the indirect assay offers the possibility to determine class-specific antibody (Voller et al., 1976; Yolken et al., 1978). However, all commercially available sera directed against bovine IgM or
IgA, that have been tested so far, contained interfering levels of antibody against rotavirus.

The results obtained with the ELISA, as carried out in this study, were positively correlated with those of the neutralizing antibody assay (Fig. 2). The rapid decline in rotavirus-specific neutralizing antibody in successive colostrum samples after calving observed by Woode and Bridger (1975) was confirmed in this study. A similar rapid decline in antibody content was found with ELISA. These observations indicate that the ELISA can be used to study the role of colostrum in rotavirus-induced neonatal calf diarrhoea.

The fact that nearly all cows studied had rotavirus-specific antibody in their colostrum, combined with the observed rapid decline of the colostrum titers after calving, may explain why rotavirus-associated diarrhoea was mainly observed after the third day of life. Moreover, these observations make it unlikely that a strong relationship may be found between the initial colostrum ELISA titer and rotavirus-associated diarrhoea in calves. On the farm that was observed for 18 months, the mean ELISA titer and the percentage of calves developing diarrhoea seemed to be negatively correlated during 1976. However, during the second quarter of 1977 the same percentage of calves developed diarrhoea as in the corresponding period of 1976, although there was a threefold difference in mean ELISA titer (Fig. 4).

On the second farm, which was followed during a restricted period in the calving season, relatively high mean ELISA titers seemed to be correlated with a delayed rotavirus excretion and a lower incidence of rotavirus-associated diarrhoea.

On the origins of the differences observed on these two farms, can only be speculated. They may be attributable to the different duration of the observation periods, the fact that one of the two farms had a clear calving season, differences in husbandry, etc.

The results reported in this study indicate that vaccination of cows can hardly be expected to prevent diarrhoea in their calves, since the initial titer of colostrum has only limited influence on the course of the disease. A more rational approach seems to be the prolonged use of first-day colostrum, as suggested by Woode et al. (1975). For testing and selecting colostrum samples for this purpose, ELISA proved to be a useful tool.

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Summary

The blocking method of ELISA for the detection and titration of rotavirus-specific antibody in colostrum is described. The results obtained were positively correlated with those of a neutralizing antibody test. On one farm colostrum samples were obtained over a period of 18 months. No relationship was found between the titer of colostrum obtained shortly after calving, and the development of rotavirus-associated diarrhoea in calves. On a second farm only samples obtained during the calving season were tested. Within this restricted period high colostral antibody titers appeared to reduce the incidence of diarrhoea among calves and to delay the onset of rotavirus excretion in the faeces. These results are discussed in relation to the rapid decline in antibody content of colostrum after calving.

References


