Survey of rotavirus infection in a dairy herd: comparison between polyacrylamide gel electrophoresis and two commercial tests.

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ABSTRACT

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A survey of rotavirus infection in a dairy herd with a history of neonatal diarrhoea was carried out. Faecal samples taken from 15 cows before and after calving as well as faeces taken from their calves daily from birth to two weeks of life were tested for rotavirus by polyacrylamide gel electrophoresis (PAGE) and compared with an ELISA and a latex agglutination commercial test. Rotavirus excretion was not detected in faeces from cows around parturition by any of the three tests. However, all of their calves shed rotaviruses during the observation period. The onset of rotavirus excretion determined by PAGE ranged from day 2 to day 8 of life (day 4.8 ± 1.8 on average) and lasted for 4 to 7 days (5.3 ± 1.1 days on average). Chi-square test showed a significant association ($P=0.0001$) between the presence of rotavirus and the altered consistency of calves faeces. All the three tests showed similar results (overall agreement 92.5%) but discrepancies were detected mainly at the beginning or at the end of the rotavirus excretion period. Results obtained with both commercial kits closely paralleled each other and parameters other than sensitivity, specificity, diagnostic accuracy or predictive values have to be considered as selection criteria.

INTRODUCTION

Neonatal calf diarrhoea is a major threat to the cattle industry due to mortality, medical costs and poor growth of calves in both dairy and beef herds. The syndrome has a great aetiological complexity and numerous pathogens have been reported in association with the disease (Woode and Bridger, 1975; Woode et al., 1976; Moon et al., 1978; Tzipori, 1981). Rotavirus is generally

accepted to be an important cause of neonatal diarrhoea in calves throughout the world with its greatest frequency and severity within the first two weeks of life (Acres et al., 1977; De Leeuw et al., 1980). Large numbers of rotavirus particles are excreted in faeces of diarrheic calves and are highly resistant to inactivation. Thus, the main source of rotavirus in an outbreak results from environmental contamination and the spread of infection can temporarily be broken by thorough cleaning and disinfection of the premises (McNulty and Logan, 1983). However, subclinically infected older calves and adult cows may play a role in the spread of infection to young calves as well as in the perpetuation of infection within a herd (Goto et al., 1986). Various methods have been developed for rapid detection of rotaviruses in faecal samples; these include electron microscopy (considered for a long time as the reference method), immune electron microscopy, counter immuno-electrophoresis, complement fixation, fluorescent antibody tests, different immunoassays using antibodies labelled with radioisotopes or enzymes (ELISA), latex agglutination and polyacrylamide gel electrophoresis (PAGE). Some of these tests are commercially available.

This paper presents the results of a longitudinal survey of rotavirus infection from birth to two weeks of life in conventionally reared calves using the PAGE technique and analyzes the relationship between rotavirus shedding and the consistency of faeces. Faecal shedding of rotavirus in their dams around calving is also studied. The PAGE results are compared with those obtained by two commercial kits, one using ELISA and the other latex agglutination.

MATERIALS AND METHODS

Herd management

The survey was carried out on a Friesian dairy farm located in northeastern Spain containing approximately 500 heifers and cows. The herd had a history of recurrent neonatal diarrhoea for previous years. Pregnant cows were vaccinated with a bacterin against *E. coli* K99+ (Imocolibov, Rhone-Merieux laboratories) approximately three weeks before the expected calving date and were usually moved to a communal house one week before calving. The calves were separated from their dam a few hours after birth and moved to a communal pen next to the cows where they remained for at least 10 days and then they were transferred to fattening units with 40–50 calves per unit. Each calf was bucket-fed its mother's colostrum during the first day of life (2 feedings, 3 litres per feeding), the first feeding being usually within 12 hours after birth, and pooled cows colostrum during the second day of life in the same conditions. Thereafter, they were fed milk replacers from an automatic feeder.

Sampling procedure

Fifteen cows, chosen on the basis of their predicted calving dates, were included in the survey. Calvings took place over the period 27 January–18 February 1991.

Faeces samples were collected from cows before calving (days 1, 3, 7 and between days 9–20 pre-partum; more samples were taken but only those cited were tested), the day of calving and after calving (days 1, 3 and 7 post-partum). Faeces samples were also obtained from the rectum of the 15 calves daily from birth (considered as day 0) to 15 days of life. At each sampling the faeces were recorded as being normal, abnormal (if not obviously diarrheic but changed with respect to consistency and/or presence of blood or mucus) or diarrheic (if fluid or profuse). Faecal score was determined by the same person for reasons of uniformity of interpretation. None of the calves with diarrhoea was treated at all or isolated from other calves.

Examination of faeces for rotaviruses

All the faeces samples (120 from cows and 240 from calves) were tested for the presence of rotaviruses by polyacrylamide gel electrophoresis (PAGE), a commercial ELISA kit and a commercial latex agglutination kit.

PAGE. The extraction of viral RNA, its resolution and staining was carried out as described by Herring et al. (1982) with minor modifications.

Briefly, faecal specimens were diluted 1:4 by weight with extraction buffer containing 1% sodium dodecyl sulfate, an equal volume of 3:2 phenol-chloroform was added and the mixture was vortexed and centrifuged for 10 min at 1200 g. The aqueous phase was removed. For electrophoresis, 40 μl of the clear supernatant were mixed with 10 μl of blue marker (25% sucrose containing 0.1% bromophenol blue) and loaded onto a discontinuous polyacrylamide gel (3% concentration for the stacking gel and 7.5% for the running gel). The gels were assembled using a mini-Protean II cell (BioRad Laboratories, Richmond, California, USA) and ran with a 50 mA running current and constant voltage for 1.5 h using a 200/2.0 model power supply (BioRad). After that, gels were fixed, developed and silver-stained. A positive control sample was always included in each gel for comparison of the segmented viral RNA migration pattern.

Commercial ELISA. The Enzygnost Rotavirus (Ag) monoclonal test (Behring Institute, Behringwerke AG, Marburg, Germany) was performed according to the manufacturer's instructions.

Briefly, faeces taken using the dispenser provided were transferred to 0.2 ml of the dilution buffer and throughly mixed. Then, 0.15 ml of the supernatant was transferred to the test plate wells coated with rabbit SA-11 rotavirus antiserum. Following incubation for 30 min at 37°C the plates were washed

3 times and 0.1 ml of antirotavirus conjugate consisting of a mouse monoclonal antibody conjugated with peroxidase were added to each well. The plates were incubated and washed again in the same conditions, 0.1 ml of the substrate-chromogen were added per well and were incubated for 30 min at room temperature protected from light. Finally, the reaction was stopped and the plates were read at 450 nm wavelength using a Multiskan spectrophotometer (Flow Laboratories). The cut-off value was calculated by adding the mean value of the negative control samples to a threshold value of 0.1 O.D. units. The retest limit was calculated by adding 0.08 O.D. units to the cut-off value. In this way results could be considered as negative (if the absorbance of the sample was less than the cut-off value), positive (if it was greater than the retest limit) and gray zone (if it was between the cut-off and retest limits).

Commercial latex agglutination. The Slidex Rota-Kit 2 monoclonal test (bioMérieux, Marcy-l'Etoile, France) was carried out according to the instructions enclosed with the kit.

Briefly, 0.2 gr of the faecal specimen were diluted in 2 ml of the buffer supplied and mixed. The emulsions were centrifuged for 10 min at 800 g and then a drop of supernatant from each specimen was placed in each of two circles on an agglutination card. A drop of reagent 1 (latex coated with monoclonal antirotavirus antibody) was added to one and a drop of reagent 2 (latex coated with rotavirus negative serum) to the other. Following mixing, the cards were rotated gently for 2 min. A positive reaction was considered when clear agglutination of the test latex with no agglutination of the control was seen. Agglutination of both control and test latex indicated a non-specific reaction. All samples were read by the same person.

Statistical methodology

In order to study the association between the variable "presence of rotavirus" and the variable "consistency of faeces", a 2×3 contingency table was done and the chi-square test was performed.

Diagnostic test results were analyzed in two ways: first, with PAGE as a gold standard and secondly, on a comparative basis with each other. In the first case, results of the commercial tests were compared with those of PAGE by using sensitivity, specificity, positive predictive value, negative predictive value and diagnostic accuracy. In the abscence of a standard, the Kappa statistic was used for comparison. The comparative measures are defined as follows:

Sensitivity (true-positive rate): is the proportion of samples in which the test is positive when PAGE is positive.

Specificity (true-negative rate): is the proportion of samples in which the test is negative when PAGE is negative.

Positive predictive value: is the proportion of samples in which PAGE is positive when the test is positive.

Negative predictive value: is the proportion of samples in which PAGE is negative when the test is negative.

Diagnostic accuracy: was determined by dividing the number of positive and negative specimens in both the commercial kit and PAGE by the total number of specimens tested.

Kappa statistic is an overall measure of agreement between two tests and is useful for measuring agreement in the absence of a standard. It compares the observed proportion of samples in which the tests agree with the proportion that would be expected to agree by chance. Kappa calculation was carried out as described by Goodman and Kruskal (1972). For interpreting Kappa values the following benchmarks can be considered: a result between 0.0 and 0.20 indicates a slight agreement, between 0.21 and 0.40 is a fair agreement, between 0.41 and 0.60 is a moderate agreement, between 0.61 and 0.80 is a substantial agreement and between 0.81 and 1.00 is an almost perfect agreement.

RESULTS

Detection of rotavirus shedding by PAGE

Rotavirus shedding was not detected in any sample taken from cows before or after parturition. All 120 samples were also negative for rotavirus when cold-ethanol precipitation of RNA was performed.

The results of rotavirus shedding in faecal samples taken from the calves are shown in Table 1. During the first two weeks of life rotavirus was detected in the faeces of all 15 calves. The presence of rotavirus was determined in 80 out of 240 samples (33%). Thirteen out of 15 calves showed a continuous shedding pattern and only two calves (calves no. 2 and 11) showed a discontinuous one.

The onset of rotavirus excretion in faeces ranged from day 2 to day 8 of life (day 4.8 ± 1.8 as a mean) and the duration of that excretion ranged from 4 to 7 days (mean 5.3 ± 1.1 days). Only one electropherotype was found in the survey and it was characteristic of group A rotaviruses.

Rotavirus shedding and consistency of calves faeces

All calves passed abnormal and diarrheic faeces along the two week observation period but no fatal cases were observed during that time (Table 1). The onset of rotavirus excretion in faeces coincided with the onset of diarrhoea or of excretion of abnormal faeces in some of the animals (clearly in calves no. 1, 4, 9, 12 and 15) but that association was quite variable. However, when every sample was considered independently of the animal and of the chronological sequence, the chi-square test showed a high association be-

TABLE 1

Rotavirus shedding pattern by PAGE and consistency of faeces in samples taken daily from 15 calves during their first two weeks of life

Day	Calf no.														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
0	−N	−N	−N	−N	−N	−N	−N	−N	−N	−N	−N	−N	−N	−N	−N
1	−N	−N	−N	−N	−N	−A	−N	−N	−N	−N	−N	−N	−A	−N	−A
2	−N	−D	+N	−N	−N	−D	−N	−D	−A	−A	−N	−D	−A	−D	−N
3	−N	−N	+N	+A	−A	+D	−N	−A	−N	−N	−N	−A	+A	−N	−N
4	+D	+N	+N	+A	−N	+D	−N	−A	−N	−N	−N	−N	+D	−A	+A
5	+D	−N	+N	+D	−A	+D	−N	−A	+N	+N	−A	−N	+A	+A	+D
6	+D	+N	+D	+D	−D	+D	−D	+D	+D	+N	−A	+A	+A	+N	+D
7	+N	−D	+A	+A	−D	+D	−D	+D	+D	+D	+D	+D	+A	+N	+A
8	+A	−A	+A	+A	+D	−D	+A	+D	+D	+A	−D	+D	+A	+D	+A
9	+A	+A	−A	−A	+D	−D	+A	+D	+A	+D	+D	+A	−N	−A	+D
10	+N	+A	−D	−A	+N	−D	+A	+D	+A	+A	+A	−N	−N	−A	−A
11	−N	−N	−N	−N	+A	−D	+D	−D	−N	−A	+A	−N	−N	−A	−A
12	−D	−D	−D	−A	−A	−A	+D	−A	−A	−A	−A	−A	−N	−N	−A
13	−D	−D	−D	−N	−D	−A	+D	−A	−N	−N	−A	−N	−A	−N	−N
14	−D	−N	−A	−N	−A	−N	−A	−A	−N	−A	−N	−N	−N	−A	−N
15	−D	−N	−A	−N	−A	−A	−A	−A	−N	−N	−A	−N	−N	−N	−N

+: presence of rotavirus. −: absence of rotavirus. N: normal faeces. A: abnormal faeces. D: diarrheic faeces.

TABLE 2

Observed and expected ( ) frequencies of the association between the variable "presence of rotavirus" and the variable "consistency of faeces" in 240 faecal samples from calves tested by PAGE

Consistency of faeces	Rotavirus		Total
	−	+	
Normal	84 (65)	14 (33)	98
Abnormal	50 (54)	31 (27)	81
Diarrheic	26 (41)	35 (20)	61
	160	80	240

+: presence of rotavirus. −: absence of rotavirus.

tween the two variables in such a way that, when a sample was positive for rotavirus, the faeces tended to be diarrheic and when a sample was negative for rotavirus it tended to be normal ($P=0.0001$) (Table 2).

Comparison between PAGE and the commercial tests

As observed with PAGE, rotavirus shedding was not detected in cows faeces taken around calving by any of the commercial tests.

TABLE 3

Comparison of results obtained by PAGE, commercial Latex agglutination (L.A.) and commercial ELISA for rotavirus detection in 240 faecal samples from calves

PAGE	L.A.	ELISA	No. of samples
+	+	+	64
+	−	G.Z.	2
+	−	−	8
+	−	+	6
−	−	+	2
−	−	−	158

+: presence of rotavirus. −: absence of rotavirus. G.Z.: gray zone (see text for details).

TABLE 4

Discrepant results obtained by PAGE, commercial latex agglutination (L.A.) and commercial ELISA tests for rotavirus detection in 240 faecal samples from calves

Test			Sample	
PAGE	L.A.	ELISA	Calf no.	Day
+	−	G.Z.	1	10
			3	9
+	−	−	2	4
			2	6
			3	2
			4	3
			7	8
			9	10
			11	7
			13	8
+	−	+	2	10
			4	9
			5	8
			6	7
			12	9
			14	5
−	−	+	11	8
			12	5

+: presence of rotavirus. −: absence of rotavirus. G.Z.: gray zone (see text for details).

When calves faeces were tested, results obtained by ELISA and latex agglutination commercial kits closely coincided with those by PAGE: the agreement between PAGE and latex agglutination was 93.33%, 95% between PAGE and ELISA, 96.66% between latex agglutination and ELISA and the overall agreement between the three tests was 92.5% (Table 3). The discrepant results fall into four categories and are shown in Table 4. Most of the discrepancies (13 samples) were observed in the first or in the last sample positive

TABLE 5

Evaluation of the performance of Latex agglutination (L.A.) and ELISA commercial kits in the detection of rotavirus in 240 faecal samples from calves relative to PAGE

Test	Samples tested by PAGE		Sensitivity (%)	Specificity (%)	P.P.V. (%)	N.P.V. (%)	D.A. (%)
	80 +	160 −					
L.A.							
positive	64	0	80	100	100	90.91	93.33
negative	16	160					
ELISA							
positive	70	2	87.5	98.75	97.22	94.05	95
negative	10	158					

P.P.V.: Positive predictive value. N.P.V.: Negative predictive value. D.A.: Diagnostic accuracy. (See text for definitions).

TABLE 6

Kappa statistic for intertest agreement between PAGE, commercial Latex agglutination (L.A.) and commercial ELISA for rotavirus detection in faeces of calves

Test	Kappa statistic		
	PAGE	L.A.	ELISA
PAGE	1.0	0.84	0.88
L.A.		1.0	0.92
ELISA			1.0

for rotavirus by PAGE in the calves rotavirus excretion period, that's to say, when the shedding pattern changed from negative to positive or vice versa and corresponded to samples negative by latex agglutination while negative (or gray zone) by ELISA (7 samples) or positive by ELISA (6 samples).

Another three discrepancies were detected in which the samples were positive by PAGE and negative by both ELISA and latex agglutination and all of them corresponded to two calves with a discontinuous shedding pattern (calf no. 2, days 4 and 6; calf no. 11, day 7). The other two discrepant results corresponded to samples positive by ELISA and negative by both PAGE and latex agglutination; one of them was detected in a calf with a discontinuous shedding pattern (calf no. 11, day 8) and the other one corresponded to the first positive sample in the rotavirus shedding pattern determined by ELISA (calf no. 12, day 5).

In Table 5 sensitivity, specificity, positive and negative predictive values and diagnostic accuracy of both latex agglutination and ELISA kits are shown considering PAGE as a gold standard. Latex agglutination showed a lower

sensitivity (80%) and a higher specificity (100%) than ELISA (87.5% and 98.75%, respectively) but the predictive values and the diagnostic accuracy were quite similar for both tests.

The Kappa statistic for each pair of tests (when no gold standard was considered) is given in Table 6. It shows an almost perfect agreement between the three tests (Kappa values between 0.8 and 1.0).

DISCUSSION

All calves monitored in our survey were shown to be infected and shed rotaviruses in faeces within the first two weeks of life. This result seems to indicate that bovine rotavirus is enzootic at this farm and it could explain its history of recurrent outbreaks of neonatal diarrhoea.

In the present study, rotavirus was detected in the faeces of some animals as early as 2–3 days of age with an increasing number of excretors thereafter. Experimental infection of calves has shown that the period between infection and detection of excretion of the virus in faeces is about two days (De Leeuw et al., 1977; Logan et al., 1979). That indicates that most calves were exposed to infection very soon after birth. Excretion of rotavirus in calves faeces has been reported before 48 hours of age but such an early detection is infrequent (Acres and Babiuk, 1978; De Leeuw et al., 1980). The delay in excretion of the virus under field conditions is probably because of the presence of specific colostral antibody within the gut lumen and the elimination of this antibody renders the calf susceptible to overt infection as suggested by De Leeuw et al. (1980). So, the early detection of rotavirus in faeces detected in the present study could be due to an inadequate feeding of colostrum.

In our study, rotavirus shedding in faeces started at 4.8 days of life on average and showed a mean duration of 5.3 days. In a similar survey, McNulty et al. (1983) reported an onset of rotavirus excretion at 6.1 days old on average in an endemically infected dairy herd and similar excretion periods have been described in both beef and dairy herds by others (Acres and Babiuk, 1978; De Leeuw et al., 1980; Goto et al., 1986). Nevertheless, the exact onset and duration of rotavirus shedding is difficult to establish in all these studies because they lack a daily sampling design.

Rotavirus excretion coincided with abnormal or diarrheic faeces in some animals and chi-square test showed a statistically significant association ($P=0.0001$) between the presence of rotavirus and the altered consistency of faeces. This suggests that rotaviruses are involved in the aetiology of this outbreak of neonatal diarrhoea although no other agents were studied. De Leeuw et al. (1980) described that rotavirus infection was associated with cases of "late diarrhoea" (diarrhoea from the 4th to the 14th day of life) whereas other agents appeared to be of minor importance, and Schusser et al. (1982) reported that, although the correlation between rotavirus excretion and clin-

ical diarrhoea in calves was poor during the first 8 weeks of life, the severest form of watery diarrhoea was limited to the first three weeks of life, where rotavirus excretion peaked. The association between rotavirus infection and abnormal or diarrheic faeces has also been reported by several authors (McNulty and Logan, 1983; Goto et al., 1986; Bellinzoni et al., 1987; Murakami et al., 1987).

The role that adult animals may play as a source of infection for calves has been a matter of controversy. In the present study, we did not detect the presence of rotavirus in any sample taken from cows around parturition by any of the three tests. Our results agree with those of several authors that have also failed to detect rotaviruses in faeces of adult animals (Dagenais et al., 1980; Scherrer et al., 1981; Sihvonen and Miettinen, 1985).

On the contrary, there have been several reports describing the excretion of rotavirus in faeces of a small number of animals with or without diarrhoea and usually over a short period around parturition (McNulty and Logan, 1983; Bellinzoni et al., 1987; Murakami et al., 1987) and it has been suggested that adult carriers might be important in the epizootiology of the infection (Woode, 1978; Crouch and Acres, 1984). Recently, Kodituwakku and Harbour (1990) reported an intermitent excretion of rotavirus in 10 asymptomatic cows throughout pregnancy although rotavirus excretion and diarrhoea were detected in only one of their calves and Goto et al. (1986) have indicated that the perpetuation of rotavirus infection within a herd may result from persistent or chronic infections in older calves and adult animals. However, it is generally accepted that once an outbreak is underway, young calves act as amplifiers of the infection and that environmental contamination is the major source of infection for newborn calves, whereas maternal infection is of minor significance (Dagenais et al., 1980; McNulty and Logan, 1983; Goto et al., 1986; Murakami et al., 1987).

The rotavirus shedding pattern obtained with the three tests was quite similar, the overall agreement being 92.5% among the three tests. Most discrepant results were positive by PAGE while negative by latex agglutination (16 samples) and negative (or gray zone) by ELISA (10 samples) and all of them except three corresponded to the first or last positive sample in the PAGE shedding pattern, when the viral content in faeces was probably low. These results seem to indicate a higher sensitivity of the PAGE test compared with that of latex agglutination and ELISA. The same explanation may be given for the discrepant results observed in calf no. 2 (days 4 and 6) and calf no. 11 (day 7) in which a discontinuous shedding pattern was detected by PAGE but we have no certainty.

The results observed in two samples (calf no. 11, day 8 and calf no. 12, day 5), in which rotaviruses were detected only by ELISA, are more difficult to explain. They could be false-positive results but if the shedding patterns of that calves are considered, the true-positive result cannot be ruled out.

The sensitivity, specificity, predictive values and diagnostic accuracy of both commercial kits used in our survey are clearly comparable and grossly parallel the results reported with several commercial and non-commercial tests (Morinet et al., 1984; Sambourg et al., 1985; Bryden, 1987; Martin and Follett, 1987; Jenkins, 1988; Thomas et al., 1988; Kok and Burrell, 1989; Molyneaux et al., 1989). To our knowledge, this is however the first time in which comparisons between different tests are made in a longitudinal survey with a daily sampling design. The overall results indicate that the three tests can be successfully used for diagnostic purposes when faeces samples from several calves are taken in the course of an outbreak of diarrhoea and that several factors such as financial means, equipment requirements, time availability, number of specimens processed or staff skills have to be considered as choice criteria.

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