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Reduction in morbidity due to diarrhea in nursing beef calves by use of an inactivated oil-adjuvanted rotavirus – *Escherichia coli* vaccine in the dam

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ABSTRACT

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An outbreak of neonatal diarrhea occurred among beef calves (2000 animals) from one large Argentinian farm in 1985. Rotavirus was detected in 78% (106/136) and enterotoxigenic *Escherichia coli* in 1.5% of the samples (2/136) obtained from sick calves. In comparison rotavirus was identified in only 1.6% (1/63) of the samples from clinically healthy calves. The rotavirus strain responsible for the outbreak was characterized as serotype 6 belonging to group A. In the following three years the protective capacity of a combined rotavirus–*E. coli* inactivated vaccine administered to the dams during the last third of the gestation period was evaluated on this farm by comparison of morbidity due to diarrhea in calves from vaccinated vs. placebo cows within the same year.

The morbidity due to diarrhea among calves from dams in the vaccinated and placebo groups was 34% and 77%, respectively in 1986; 23% and 47% in 1987, and 15% and 34%, in 1988. In 1987 morbidity of diarrhea in calves born from vaccinated heifers was 54% and 74% in calves from placebo heifers. In 1988 morbidity from diarrhea was 41% and 54%, respectively among calves in these two groups. In all experiments, calves from heifers showed significantly greater morbidity than calves from cows. Differences in diarrhea morbidity between the vaccinated and placebo groups were statistically significant ($P<0.05$). Additional studies showed that the diarrhea had a significant influence ($P<0.05$) on the average live weight of the calves at weaning (5 to 7 months) with an average weight loss of 7.8 kg per calf among the calves affected with diarrhea.

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INTRODUCTION

Neonatal diarrhea is one of the major disease syndromes affecting calves in different countries of the world and is a cause of important economic losses. Diarrhea is defined as having a complex multifactorial etiology, influenced by infectious, nutritional, and environmental factors as well as management practices (Roy, 1980; Tzipori, 1981; McNulty, 1983; Acres, 1985). Among these factors, infectious agents are considered first degree causative agents in this syndrome with rotavirus as a major cause of neonatal diarrhea in bovine and other animal species (Tzipori, 1981; McNulty, 1983; Simhon, 1985).

In Argentina, previous studies documented diarrhea as the principal cause of morbidity and mortality among calves younger than 45 days of age (Cornaglia et al., 1989). The high morbidity rate (90–100%) observed for this disease and the absence of a practical and effective treatment has necessitated the implementation of preventive measures based on the use of specific vaccines (DeLeeuw et al., 1980; Snodgrass et al., 1980; Eichhorn et al., 1983; Saif et al., 1983, 1984; Saif, 1985; Dauvergne et al., 1983).

The purpose of this work was first to survey for selected infectious agents known to cause diarrhea (rotavirus, *E. coli* and *Salmonella* spp.) present in the stools of beef calves less than 45 days of age and examine their association with diarrhea. Secondly we analyzed the protective capacity of a combined rotavirus–enterotoxigenic *E. coli* (ETEC) inactivated vaccine administrated to the dams as a method for the prevention of neonatal diarrhea in the nursing calf. Evaluation of vaccine efficacy was based on a comparison of the morbility rates of calf diarrhea in the vaccinated vs. placebo animals within each year of the study.

MATERIALS AND METHODS

Selected area

For this study, a 2000 ha farm representative of others in Argentina was selected, which performed extensive breeding of cattle for meat production with 2000 Aberdeen Angus×Charolais females. It was located in the middle of the Pampean region, in the Province of Buenos Aires, Argentina. The cows received natural service in the field during the months of October, November and December, giving birth during the months of July, August and September, the winter period (characterized by temperatures below 0°C and frequent rain-fall). The data obtained previously from this farm indicated that in the previous four years, severe outbreaks of diarrhea were observed in calves less than 45 days of age, with morbidities that ranged from 70 to 90% and mortalities between 1 and 2%.

Sampling and clinical observations

During the 1985 (pre-vaccine) and 1986 sampling periods, stools were collected from calves with diarrhea ($n=136$ and $n=69$ in 1985 and 1986, respectively) and known clinically healthy calves ($n=63$ in 1985 and $n=238$ in 1986). The age of the animals ranged between 0 and 6 weeks. Selected samples were analyzed by the following tests including ELISA, virus isolation and serum neutralization as described in the subsequent sections. Only a few samples were analyzed by these tests in the 1987 and 1988 experiments. All calves between 0 and 6 weeks of age in all experiments were observed daily for signs of diarrhea.

Enzyme linked immunosorbent assay (ELISA)

For rotavirus detection the samples were processed by the double antibody sandwich ELISA technique (Cornaglia et al., 1989). Briefly, hyperimmune rabbit anti-rotavirus serum (Lincoln strain) was used as the capture antibody and hyperimmune bovine anti-rotavirus serum (Lincoln strain) as the secondary antibody. The detector antibody was rabbit anti-bovine IgG conjugated to alkaline phosphatase.

Virus isolation

Samples ($n=106$ in 1985 and $n=44$ in 1986) were inoculated in duplicate into monolayers of primary fetal bovine kidney (FBK) cells. Rotavirus detection was carried out 24 h post inoculation (p.i.) by direct immunofluorescence (IF) using antiserum prepared in a calf against the Lincoln strain of bovine rotavirus. Rotavirus strains from stools were adapted to serial passage in Rhesus monkey kidney (MA-104) cells and passaged until titers higher than 10^5 $TCID_{50}$/ml were obtained.

Rotavirus serotypes

A one-way seroneutralization test (SN) was done in MA-104 cell monolayers grown in 96-well microtiter plate using a constant antiserum, varying virus method (Ojeh et al., 1984). Serial ten-fold dilutions of several rotavirus positive cell culture-passaged field strains ($n=15$ in 1985 and $n=9$ in 1986) and of the Lincoln rotavirus strain (serotype 6) were tested against 20 seroneutralizing units (SNU) of an anti-rotavirus serotype 6 reference serum (Lincoln strain)*. The viral titers were revealed 48 h p.i. by direct IF and expressed as the reciprocal of the last viral dilution which was 100% neutralized by the antiserum.

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Polyacrylamide gel electrophoresis (PAGE)

Viral ds RNA was extracted from diarrheic ($n=106$) and nondiarrheic stools ($n=1$) from 1985 samples using the method described by Nichols et al. (1983). In samples from the subsequent three experiments only selected rotavirus positive samples were analyzed. The polyacrylamide gels (7.5%) were prepared and run according to the method described by Laemmli (1970) and developed with silver.

Enterotoxigenic E. coli *(ETEC)*

All stool samples collected in 1985 and 1986 were streaked onto blood agar and MacConkey's agar plates and incubated for 24 h at 37°C. The biochemical indentification (Lennette, 1985) and the enterotoxigenicity tests (Desmettre et al., 1982) were then performed, which consisted of the detection of the K99 antigen (Guinee et al., 1977) and the production of heat-stable enterotoxin (Dean et al., 1972), respectively. K99 antigen was identified by direct IF *.

Salmonella

The same samples as for *E. coli* isolation were also inoculated into selenite broth and later streaked onto Salmonella–shigella agar and MacConkey's agar, and incubated for 24 h at 37°C (Lennette, 1985).

Experimental vaccine

A combine rotavirus–ETEC vaccine with oil adjuvant was used. Each 5 ml dose contained 1.5 ml of serotype 6 rotavirus native strain (isolated during the outbreak which occurred in this herd in 1985, and propagated 17 times on FBK cells) with a titer of 10^7 $TCID_{50}$, and 1 ml of a bovine K99 ETEC B41 strain** with a concentration of 10^{11} bacteria/ml and with a K99 antigen titer of 1/64. Both antigens were inactivated with 5% formalin (final concentration) and formulated in equal quantities with Marcol/Arlacel oil*** adjuvant. A placebo vaccine was administered consisting of the oil adjuvant and mock-infected culture medium prepared as a control. The vaccine was administered by the subcutaneous route in two doses within 30 days intervals to pregnant females in the last third of the gestation period.

Experimental groups

Experiment 1 (year 1986)

For this experiment, 1024 pregnant females were included and assigned to one of three experimental groups. These groups were: Group 1 ($n=677$) in

*FITC Labelled rabbit anti *E. coli* 11 K99.
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which approximately 50% of the females were vaccinated and the remaining 50% received placebo vaccination; Group 2 ($n=159$) in which 100% of the females were vaccinated; and Group 3 ($n=188$) in which 100% of the females received placebo vaccination (control group).

Experiment 2 (year 1987)

This experiment was conducted using a total of 1473 pregnant females assigned to one of four treatment groups (Groups 4 and 6, 100% vaccinated heifers ($n=219$) or cows ($n=593$) respectively and Groups 5 and 7, 100% placebo heifers ($n=226$) and cows ($n=435$) respectively). The efficacy of the treatment in cows and heifers was also compared (Groups 4 and 6 vs. Groups 5 and 7).

Experiment 3 (year 1988)

This experiment was conducted on 1556 animals distributed among 5 experimental groups: Group 8, heifers vaccinated with one dose ($n=331$); Group 9, placebo heifers ($n=149$); Group 10, cows vaccinated with one dose ($n=303$); Group 11, cows vaccinated with two doses ($n=524$); and Group 12, cows vaccinated with placebo ($n=249$).

Calf weights

At weaning, 328 animals between 5 and 7 months of age were weighed individually (Experiment 1, 1986). The two weight groups were as follows: 99 calves which had clinical diarrhea in their first month of life; and 209 clinically healthy calves.

Statistical analysis. The results obtained were analyzed by χ^2 with confidence levels set at 95%.

RESULTS

Diagnosis (year 1985)

The morbidity due to diarrhea during 1985 was 95% (300 of 315 calves studied) with an average age at onset of 14 days. Of the 136 samples from diarrhea analyzed, rotavirus was detected in 106 (78%) of the samples and ETEC in only two (1.5%). However, of samples obtained from clinically healthy animals ($n=63$), rotavirus was detected in only 1 (1.6%) and ETEC in none. Except for two samples in which enterovirus was detected by virus isolation techniques, no other enteric agents were identified. Of the three techniques used, rotavirus was detected by ELISA in 102/106 samples (96%), by PAGE in 96/106 (91%) and by cell cultured immunofluorescence in MA 104 cells in 83/106 (78%). Only minor differences were observed in the ds RNA migration patterns by the PAGE technique for the different samples

analyzed, all of which belonged to rotavirus electropherogroup A (Fig. 1, lanes A–C). All 15 strains adapted to cell culture were characterized as belonging to serotype 6 by a one-way seroneutralization test.

Prevention

Total mortality in calves in 1985 and previous years was 1 to 2% and no differences in mortality between calves born from vaccinated or placebo dams were detected during the consecutive years.

Experiment 1 (year 1986)

Morbidity values for the three different experimental groups (Experiment 1) are presented in Table 1. The morbidity observed in Groups 1 (50% vaccinates) and 2 (100% vaccinates) was 28% and 34%, respectively for the calves from vaccinated dams. Among the calves from the placebo dams, morbidity was 35% in Group 1 (50% placebos) and 77% in Group 3 (100% placebos) ($P<0.05$). The total diarrhea morbidity in the herd was 40%.

Rotavirus was detected in 61% of the samples obtained from diarrheic calves (42/69) in the vaccinated (14/24) and placebo (28/45) groups; and only in 1% (2/151) of the clinically healthy animals. No other infectious agents associated with diarrhea were detected using the assays outlined earlier. The average age of diarrhea presentation was 16 days in calves in the placebo groups, showing a significant difference ($P<0.05$) compared to calves in the vaccinated groups (26 days). Similarly the average age of the diarrheic calves in which rotavirus was detected was significantly greater ($P<0.05$) in the vaccinated groups (31 days) than in the placebo groups (17 days).

Rotavirus strains present in the samples obtained from diarrheic calves,

TABLE 1

Summary of morbidity due to diarrhea observed in calves from vaccinated[1] or placebo dams. Experiment 1, year 1986

Group[2]	Vaccinated No. with diarrhea/total	%	Placebo No. with diarrhea/total	%	Total No. with diarrhea/total	%
1	90/325	28	122/352	35	212/677	31
2	54/159	34			54/159	34
3			144/188	77	144/188	77
Total	144/484	30	266/540	49	410/1024	40

[1]The mothers were vaccinated during the last third of the gestation period with two 5 ml doses of rotavirus-ETEC-adjuvanted vaccine given with a 30 day interval, via the subcutaneous route.

[2]Group 1: Approx. 50% vaccinated and 50% placebo dams; Group 2: 100% vaccinated dams; Group 3: 100% placebo dams.

[3]Significant differences ($p<0.05$) in calf diarrhea morbidity observed between Groups 1 and 3; 2 and 3.

born from vaccinated and placebo dams showed similar dsRNA migration patterns which differed from the pattern for the vaccine strain (Figure 1, lanes A,D). Only serotype 6 rotavirus ($n=9$) was detected which concurred with results obtained during 1985. At weaning, the median weight of the calves that developed diarrhea during their first month of life was 161.4 kg and in the clinically healthy animals was 169.2 kg. This 7.18 kg difference in average weaning weights was statistically significant ($P<0.05$).

Experiment 2 (year 1987)

The results are summarized in Table 2. The morbidity in the vaccinated groups was significantly lower ($P<0.05$) than that seen in the placebo groups in the same animal category (cows or heifers). The morbidity in the calves born from vaccinated cows was significantly lower than that registered in the calves born from heifers ($P<0.05$), when vaccinated animals were compared with each other and controls within themselves. The total morbidity of diarrhea observed in the herd in this year was 34%. By PAGE, the dsRNA migration pattern of a rotavirus strain (from a calf of a placebo dam) from this year was similar, if not identical to the pattern of the vaccine strain (Fig. 1, lanes A and E).

Experiment 3 (year 1988)

Results are shown in Table 3. Morbidity in calves from vaccinated cows was significantly lower ($P<0.05$) than that registered in calves from placebo cows, but no significant difference between calves from vaccinated (41%)

TABLE 2

Summary of morbidity due to diarrhea in calves born from vaccinated[1] or placebo cows or heifers. Experiment 2, year 1987

Group[1]	Treatment	No. with diarrhea/total[3]	%
Heifers 4	Vaccine	118/219	54
5	Placebo	168/226	74
Cows 6	Vaccine	71/593	12
7	Placebo	143/435	33
4 & 6	Vaccine	189/812	23
5 & 7	Placebo	311/661	47
Total		500/1473	34

[1]The mothers were vaccinated during the last third of the gestation period with two 5 ml doses of rotavirus-ETEC-adjuvanted vaccine given within a 30 day interval, via the subcutaneous route.
[2]Group 4: vaccinated heifers; Group 5: placebo heifers; Group 6: vaccinated cows; Group 7: placebo cows.
[3]Significant differences ($P<0.05$) in calf morbidity observed between groups 4 and 5; 6 and 7; 4 and 6; and 5 and 7.

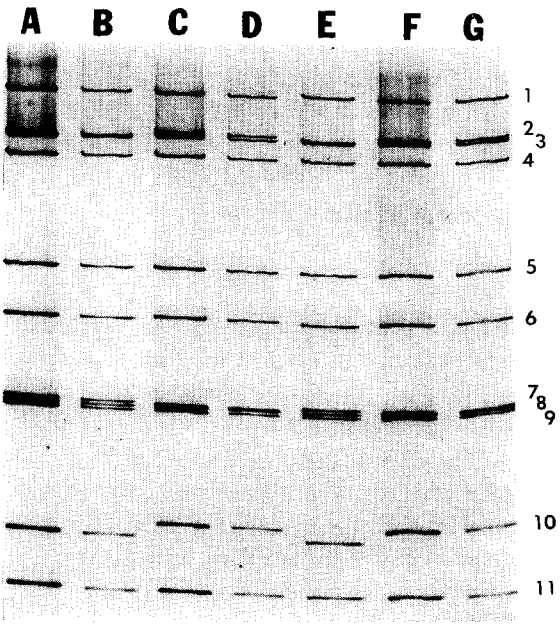

Fig. 1. Comparison of the electrophoretic migration patterns of representative rotavirus double-stranded RNA preparations by PAGE. (A) vaccine strain; (B) 7/17/85 strain; (C) 7/22/85 strain; (D) 8/9/86 strain; (E) 7/1/87 strain; (F) 8/15/88 strain; and (G) 8/31/88 strain. Numbers to the right designate genome segments.

TABLE 3

Experiment 3: summary of morbidity due to diarrhea in calves born from vaccinated[1] or placebo cows or heifers. Experiment 3, year 1988

Group[2]	Treatment	No. with diarrhea/total[3]	%
Heifers 8	Vaccine	136/331	41
9	Placebo	81/149	54
Cows 10	Vaccine	17/303	6
11	Vaccine	18/524	3
12	Placebo	53/249	21
8+10+11	Vaccine	171/1158	15
9 & 12	Placebo	134/398	34
Total		305/1556	20

[1]The mothers were vaccinated during the last third of the gestation period with two 5 ml doses of rotavirus-ETEC-adjuvanted vaccine given with a 30 day interval, via the subcutaneous route.
[2]Group 8: heifers, one vaccine dose; Group 9: placebo heifers; Group 10: cows, one vaccine dose; Group 11: cows, two vaccine doses; Group 12: placebo cows.
[3]Significant differences in calf morbidity ($P<0.05$) were observed between Groups 8 and 10, 10 and 12, and 11 and 12. Differences in calf diarrhea morbidity between Groups 8 and 9, 10 and 11 were not significant ($P<0.05$).

and placebo heifers (54%) was observed. No significant differences were observed ($P<0.05$) between Group 10 (cows vaccinated with one dose) and Group 11 (cows vaccinated with two doses) which presented a morbidity of 6% and 3%, respectively. The total morbidity for the farm was 20%, the lowest obtained among the three experiments. The two rotavirus strains showed similar if not identical dsRNA migration patterns in PAGE (Fig. 1, lanes F and G), but differed from the pattern for the vaccine strain (Fig. 1, Lane A).

DISCUSSION

Rotavirus is a major etiologic agent of bovine neonatal diarrhea, being on some occasions associated with other infectious agents (Tziopori, 1981). The clinical and epidemiological data (age of presentation of diarrhea, morbidity rate and season of the outbreak) registered in the experimental farm during 1985, added to laboratory results, indicated that rotavirus was a major etiological agent associated with diarrhea outbreaks in this herd.

The techniques used in this study failed to detect atypical rotaviruses (none identified by PAGE), other cytopathogenic viruses, and known bacterial agents as etiological agents. Even though ETEC was identified in the study population, its low frequency, younger presentation age and lack of purity in the cultures ruled out this microorganism as the major causative agent (Acres, 1985).

The 96%, 91% and 78% sensitivity values for the ELISA, PAGE and IF techniques, respectively, for the detection of rotavirus are in accordance with those observed by other authors (Cornaglia et al., 1989; Ellens et al., 1978). These results support the use of ELISA as the test of choice for field diagnosis of rotavirus.

Different vaccination strategies have been suggested for prevention of diarrhea. The stimulation of active immunity by vaccinating calves by the oral route with attenuated virus has not shown encouraging results (Acres et al., 1976; Burki et al., 1983). Furthermore, this is an impractical method in systems of extensive breeding with a large number of animals such as the groups in this study. On the other hand, immunization of dams during the last third of the gestation period with attenuated or inactivated vaccines with the aim of enhancing passive immunity has shown varied but promising results (Snodgrass et al., 1980; Eichorm et al., 1983; Saif et al., 1983, 1984; Saif, 1985; Dauvergne et al., 1983).

In the present work, an inactivated rotavirus-ETEC vaccine in oil adjuvant, administered to pregnant cows was tested in order to evaluate its efficacy in the prevention of diarrhea. The progressive decrease of morbidity observed in the vaccinate groups in the three years of evaluation suggests that partial and repetitive vaccination of the population had a negative influence on the amount of virus shed in the field, reducing the possibility of a massive infec-

tion of younger highly susceptible animals. This observation would explain the lack of a significant difference between morbidity indexes among experimental groups vaccinated by the 50 or 100% scheme (Experiment 1, Table 1). Other investigators (McNulty et al., 1987) also showed reduced diarrhea in conventional calves supplemented with colostrum and milk from vaccinated cows.

The higher morbidity registered in calves born from vaccinated or unvaccinated heifers compared to that observed in calves born from the corresponding groups of cows indicates possible differences in the quality or quantity of antibodies passively transferred via mammary secretions. This may be due to a deficiency of the immune status in the animals giving birth for the first time, in the antibody content of their colostrum and milk or in their milking ability (Gay, 1983). However the total results obtained in Experiment 3 (Table 3) following the administration of a single dose of vaccine to cows indicates that this scheme would be efficient in an adult population previously exposed to rotavirus as a preventive treatment for the passive control of rotavirus disease.

Even though the rotavirus detection rate was similar in Experiment 1 in the stools of diarrheic animals born from vaccinated or placebo mothers, the average age of presentation with diarrhea was noticeably different: 31 and 17 days respectively. This observation might indicate that the difference in the average age of diarrheic calves in the vaccinated versus placebo groups could be due to a drop in antibody levels passively transferred in the later milk, as was observed by previous investigators (Snodgrass et al., 1980; Saif et al., 1983; Saif, 1985). The possibility that the presence of rotavirus in calves of vaccinated dams was due to atypical rotaviruses or another serotype of group A different from the vaccine serotype (serotype 6) was considered unlikely due to the results obtained by PAGE and seroneutralization tests which showed similarities among all the strains detected throughout the experiments. However, only a percentage of the specimens were analyzed by this test. The fact that the average age of presentation in both animal groups in which a definite infectious etiology was not identified did not overlap entirely with the average age of rotavirus associated diarrhea would suggest the existence of other agents on the farm capable of causing diarrhea in calves between the second and third weeks of life.

CONCLUSIONS

Considering that any preventive program introduced into a herd should have an economic and positive impact on production, its has been shown that vaccination of the dams during the last third of the gestation period, decreases the diarrheal morbidity in the calves and has a significant positive influence on the average weight gains of the calves at weaning.

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