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Efficacy of an inactivated oil-adjuvanted rotavirus vaccine in the control of calf diarrhoea in beef herds in Argentina

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We have assessed the potency of an inactivated oil-adjuvanted rotavirus vaccine in beef herds in Argentina. Two different vaccine trials were conducted. In a small-scale experimental trial, involving 21 pregnant cows (13 vaccinated and eight unvaccinated controls), a significant increase in neutralizing antibody titres against different serotypes of bovine rotaviruses was found in both the colostrum and serum of vaccinated cows compared with that of unvaccinated controls. Seven days after birth, half of the calves born to vaccinated dams or to control cows were challenged with live virulent virus whereas the other half of both groups were left in contact with the infected calves in order to mimic a natural field challenge. Although no statistically significant differences in the rate of protection were observed among the different groups of animals, a larger number of vaccinated calves were protected in comparison with their controls, particularly where animals in contact with infected calves were concerned. Secondly, a large-scale field trial was carried out in 17 beef herds involving a total of 4066 vaccinated pregnant cows. In 11 farms morbidity and mortality in calves from vaccinated cows were compared with historical data from the previous 3 years at the same locations. In the other six herds, control groups were used to compare data of the same year: 1540 cows were vaccinated and 2700 were left as controls. Taking into account the previous and current incidence of diarrhoea, morbidity and mortality were significantly reduced in 16 of the 17 beef herds tested. Vaccine effectiveness was also evident in farms where other enteropathogens such as cryptosporidium and coronaviruses were present, together with rotavirus.

Keywords: Rotavirus; cattle; diarrhoea; oil-adjuvanted vaccine

Introduction

Diarrhoea in newborn calves is a syndrome in which several causative agents (acting separately or associated in different ways) may be involved. Surveys carried out in different countries have shown that rotaviruses, coronaviruses, enterotoxigenic *Escherichia coli*, *Salmonella* spp. and cryptosporidium are the pathogens chiefly responsible for outbreaks of calf diarrhoea¹⁻⁴.

The control of diarrhoea caused by different pathogenic microorganisms should be possible by the development of preventive strategies through the use of appropriate vaccines⁵, in addition to the general measures currently used, such as management, nutrition and chemotherapeutics. To be effective, these vaccines must be designed rationally and based on the impact that the different enteropathogens have in a definite geographical region; therefore single agents or appropriate combinations of them should be components of these vaccines. Other relevant factors in achieving effective vaccination are the type of vaccine (live attenuated or inactivated virus) the formulation, and the route and timing of immunization(s). Calves can be protected against rotavirus and enterotoxigenic *E. coli* by passive immunization, taking

advantage of the lactogenic immunity stimulated by maternal vaccination or directly by feeding calves with hyperimmune colostrum obtained from vaccinated dams^{6,7}. In contrast, attempts to stimulate active immunity in newborn calves by oral vaccination with live attenuated virus have been unsuccessful so far^{8,9}. With regard to the formulation of the vaccine, the most relevant factor is which type of adjuvant to use. When the target for vaccination is the pregnant cow, it has been demonstrated that oil-based adjuvants are more effective than alhydrogel to enhance colostrum and milk antibody titres, at least against *E. coli*, rotavirus and coronavirus^{10,11}.

In Argentina, neonatal diarrhoea is responsible for important economic losses in beef and dairy herds; morbidity can reach 90-100% whereas mortality ranges between 1% and 20%. Rotavirus is the major agent associated with diarrhoeal problems in Argentinian beef herds¹². For that reason, after several years of epidemiological studies, it was decided to develop and test an inactivated oil-adjuvanted vaccine with the aim of controlling diarrhoea in beef and dairy herds in Argentina.

We now report the results of two trials performed with the experimental vaccine. The first trial was a challenge experiment performed in confinement and involving 21 cows; the second was an open field trial involving 4066 vaccinated cows in 17 different beef herds. The results showed that the oil-adjuvanted rotavirus vaccine tested was effective in the control of calf neonatal diarrhoea in Argentina.

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Materials and methods

Viruses

The following rotavirus strains were used throughout this work:

Strain T67. This was isolated from a diarrhoeic calf in Argentina (1985) and was adapted to grow in MA-104 cells; it was cloned four times by limited dilution after the sixth passage. According to its antigenic characteristics this strain was classified as serotype 6 (see *Table 1*).

Strain T18. This strain, isolated from a diarrhoeic calf in Argentina (in 1986), was adapted to grow in MA-104 cells and was cloned three times. It was selected for this work because it is one of the few local strains that does not cross-react with the bovine prototype strain, UK.

Strain UK. This was kindly provided by Dr D.R. Snodgrass (Moredun Research Institute, Edinburgh, Scotland, UK). It was passaged 25 times in MA-104 cells, cloned three times and classified as serotype 6 (Ref. 13).

Strain B223. This bovine strain, isolated in Iowa, USA and kindly provided by Dr G. Woode (Texas, USA), was passaged nine times in MA-104 cells and cloned three times. Like the local strain T18, this strain did not cross react in neutralization assays with the NCDV prototype strain¹⁴.

Antisera production

Hyperimmune antisera were prepared in seronegative guinea pigs by injecting intramuscularly 10 µg of the corresponding purified virus emulsified with an equal volume of Freund's complete adjuvant. Two more injections were given subsequently with the same quantity of virus but mixed with Freund's incomplete adjuvant. Sera were collected one week after the last inoculation and monitored by ELISA and neutralization assays.

Neutralization assays

The assays were carried out as described by Gerna *et al.*¹⁵ Briefly, 50 µl virus, diluted with medium to give 200 focus-forming units (f.f.u.) per well, were mixed with 50 µl twofold serial dilutions of the antisera to be tested. Mixtures were incubated for 90 min at 37°C and inoculated on top of confluent monolayers of MA-104 cells growing in 96-well microtitre plates. The plates were incubated for 14–16 h. After fixation with acetone, cells were stained by the IPA technique¹⁵. The neutralizing titre of each serum was expressed as the reciprocal of the highest dilution that gave 80% reduction in the number of the stained cells compared with control wells. Homologous control viruses were included in the test for each serum.

Table 1 Antigenic relationship between bovine local and prototype strains

Rotavirus strain	Neutralizing titre ^a			
	UK	NCDV	T18	T67
UK	25 600	6400	100	6400
NCDV	6400	6400	100	6400
T18	100	200	3200	100
T67	12 800	3200	100	12 800

^aExpressed as the reciprocal of the highest dilution that gave 80% reduction in stained cells compared with controls (see Materials and methods)

Vaccine

The vaccine was produced with the local rotavirus strain, T67. The virus was grown in monolayers of MA-104 cells. After complete cytopathic effect, cellular fluids containing debris and virus were clarified by centrifugation (5000g for 10 min), inactivated with 0.5% formalin (48 h at 4°C), and emulsified with an equal volume of oil adjuvant comprising a mixture of 90% Marcol 52 and 10% Montanide 888 (Trademarks of Seppic, France).

The innocuity of each inactivated antigen preparation was tested by observation of any possible cytopathic effect after three serial passages in MA-104 cell monolayers.

Small-scale experimental trial

Thirteen pregnant Hereford heifers were vaccinated twice subcutaneously with 2 ml of the oil vaccine 60 and 30 days before expected delivery. Eight heifers remained as unvaccinated controls. Calves were nursed by their mothers throughout the experiment; on the seventh day of life half of the calves of both groups were injected orally with 10⁶ TCID₅₀ of the T67 strain. The uninoculated calves were kept in close proximity to those inoculated in order to evaluate the possible occurrence of natural transmission of challenge virus, as might happen in the field ('field exposure'). Calves were examined daily for 40 days and were considered diarrhoeic if animals excreted loose or watery faeces for at least 2 days⁶. Daily faeces samples were examined for rotavirus, coronavirus, cryptosporidium, enterotoxigenic *E. coli* and *Salmonella* as described by Snodgrass *et al.*³.

Cows were bled before first and second vaccination, at calving and at 30 days after delivery. Colostrum and milk samples were collected at calving and at 7, 14 and 30 days postcalving. Rotavirus-neutralizing antibodies were assayed in each sample, as described previously¹⁵. Neutralizing antibodies against the UK and T67 strains (serotype 6), the B223 strain and the T18 local strain, were determined in colostrum (first milking). In addition, neutralizing antibodies against the T67 strain were determined in the milk on various days post-calving.

Large-scale field trial

Seventeen beef herds were selected to perform the field trials, the criterion being presentation of calf diarrhoea problems during ≥3 years before this study. The 17 beef herds selected for the vaccine trial were located in the provinces of Buenos Aires and Córdoba, comprising a considerable area of the principal breeding region of the country.

These herds had previously been part of an extensive epidemiological study (involving a total of 62 herds) performed by our laboratory to determine the aetiology of the high incidence of neonatal diarrhoea in this region (R.C. Bellinzoni *et al.*, unpublished results). In 11 herds the results were compared with historical controls and in the other six herds approximately two-thirds of the cows were left as unvaccinated controls. A total of 4066 cows were vaccinated once, as described above, 1 month before the onset of calving. In four of those herds, unvaccinated cows were separated from the vaccinated animals. In the other two herds, both groups were mixed; in this case, the animals in the unvaccinated group were selected randomly. All the cows involved in the experiment were correctly identified and each one was feeding its own calf.

Furthermore, management of the herds was not disturbed by participation in the trial. The experiment was carefully controlled by trained veterinarians in close cooperation with farm personnel. During weekly visits to the herds, information about diarrhoeic animals was recorded. The trial lasted until each calf was 40 days old. In all cases, diarrhoea was defined as loose or watery faeces, a situation that usually called for treatment of the diseased animals.

In some herds, faecal samples were taken from diarrhoeic calves the year before the trial (1986) and during the present trial (1987), and were examined for rotavirus, coronavirus, cryptosporidium, *Salmonella* and ETEC as described previously³.

Results

Experimental trial

As shown in Table 2, in the vaccinated group only two of seven challenged calves, and none of those kept in close contact with them, developed diarrhoea, whereas in the control group, three of four inoculated animals and two of four uninoculated animals developed diarrhoea.

Virus excretion did not differ in the inoculated calves from both groups (vaccinated and unvaccinated), but differences were observed in close-contact controls (Table 2). All rotaviruses isolated from positive faeces showed identical genomic RNA patterns to the T67 strain used for vaccine production and challenge (data not shown).

The results shown in Table 2 suggest a higher rate of protection in vaccinated animals compared with unvaccinated controls; however, because of the small number of animals involved, no significant differences were noted on statistical analysis of the data. Other difficulties with this type of experiment are, first, assessment of the correct dose of virus to be used for the challenge, and second, the

Table 2 Rotavirus excretion in faeces and diarrhoea from challenged calves

	Vaccinated group		Control group	
	Inoculated calves	Uninoculated calves	Inoculated calves	Uninoculated calves
Excreting rotavirus/ in group	6/7	3/6	3/4	4/4
With diarrhoea/ in group	2/7	0/6	3/4	2/4

Table 3 Colostrum neutralizing titre of vaccinated cows against different bovine serotypes

Strain	Serotype ^a	Neutralizing titre ^b	
		Control group (n = 8)	Vaccinated group ^c (n = 13)
UK	6	925 (100–1600) ^d	20 800 (6400–51 200) ^d
T67	6	1300 (50–3200)	9 600 (6400–25 600)
B223	Non-6 (unclassified)	1400 (400–3200)	8 200 (800–12 800)
T18	Non-6 (unclassified)	1425 (100–3200)	14 800 (1600–25 600)

^aRef. 13; ^bexpressed as the reciprocal of the highest dilution that gave 80% reduction in stained cells compared with controls (see Methods section); ^cvaccinated with strain T67; ^dmeans of each group, with ranges in parentheses

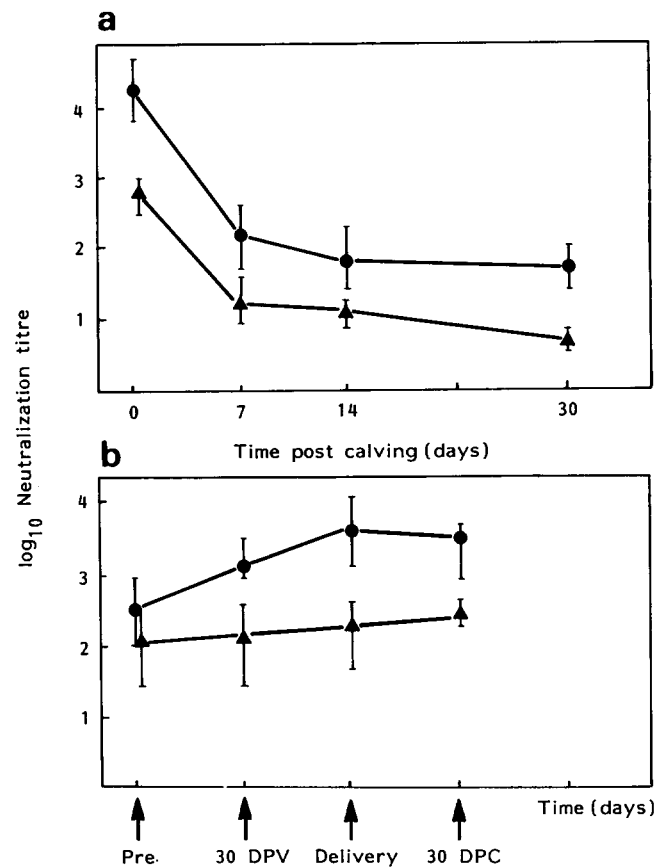


Figure 1 Neutralizing titre of colostrum milk and sera of vaccinated and control cows. Colostrum, milk and sera samples were obtained from cows involved in the small field trial at the times indicated. The level of neutralizing antibodies against strain T67 in each sample were determined as indicated in methods. The values are the mean of 13 (vaccinated group) or 8 (controls) animals assayed. Vertical bars at each point indicate the variation among individual cows. Pre: preimmune sera from the day of first vaccination; 30 DPV: 30 days postvaccination and second vaccination; 30 DPC: 30 days postcalving. a, Colostrum and milk samples; b, serum samples; ●, vaccinated; ▲, control

inability to ensure that all the unchallenged animals kept in close proximity to the infected calves received a pathogenic dose of the virus⁶.

Coronavirus, cryptosporidium or ETEC were not detected in any of the diarrhoeic samples analysed.

Aspects of the immunological status of vaccinated animals were also analysed. As shown in Figure 1, compared with controls, vaccinated cows showed significantly higher neutralizing antibody levels against rotavirus in serum, colostrum and milk until at least 30 days after calving. In addition, the titres of colostrum neutralizing antibodies were sufficiently high to neutralize other strains of rotavirus as well. As can be seen in Table 3, colostrum from cows vaccinated with a strain belonging to serotype 6 also showed neutralizing activity at similar titres against heterologous serotypes.

Field trial

Table 4 summarizes the results obtained from the large-scale field trial. In the case of herds A–F, morbidity and mortality data obtained from the last 3 years were compared with data obtained during the current trial. No significant differences were detected in the incidence of diarrhoea and mortality in the contemporary control

Table 4 Evaluation of a rotavirus vaccine in a cattle field trial

Farm ^a	Previous data			Vaccinated group			Control group ^d		
	Diarrhoeic calves ^b (%)	Dead calves ^b (%)	Enteropathogens ^c	No. of cows (n = 4066)	Diarrhoeic calves (%)	Dead calves (%)	No. of cows (n = 2700)	Diarrhoeic calves (%)	Dead calves (%)
A	80	2	R, Cr	140	5	0	250(S)	40	2
B	75	12	ND	400	20	0	800(S)	70	10
C	40	5	ND	250	20	2	200(S)	40	5.6
D	40	4.5	ND	350	3	0	250(S)	38	7.6
E	20	5	ND	100	5	0	500(M)	20	3
F	15	4	ND	300	2	0	700(M)	6	0
Mean	(45)	(5.4)			(9.2)	(0.3)		(35.7)	(4.7)
G	70	4	R, Cr	120	20	0			
H	70	4	R, Cr	300	15	0			
I	60	3	R, Co	100	4	0			
J	50	6	ND	50	0	0			
K	40	15	R, Co, Cr	80	0	0			
L	30	5	R, Co	95	3	0			
LL	30	0	R, Co, Cr	120	5	0			
M	24	3	ND	940	0.5	0			
N	20	2	R, Co	269	0	0			
O	20	3	ND	170	0	0			
P	50	15	R, Co, Cr,	282	50	2			
Mean	(42.2)	(5.3)			(8.9)	(0.2)			

^aIn farms A-F, data relating to the incidence of diarrhoea and mortality in calves born from vaccinated cows were compared with data obtained from an unvaccinated control group as well as with data from the last 3 years at the same farms. In two farms (E and F), controls were mixed (M) with vaccinated animals; in the other four farms (A-D), control animals were separated (S) from the vaccinated areas. In farms G-P, data were compared only with the data of the last 3 years at the same farms; ^bmean of the last 3 years; ^centeropathogens detected the year before vaccination: R, rotavirus; Co, coronavirus; Cr, cryptosporidium; ^dS, controls kept apart from vaccinated animals; M, control and vaccinated animals kept in close proximity

groups (35.7 and 4.7% respectively) when compared with the data of the last 3 years (45 and 5.4% respectively) suggesting that, in general, the epidemiological situation did not change significantly in those herds. Thus (apart from herd F which showed a low previous incidence of diarrhoea and mortality), vaccination of the cows led to an important reduction in the incidence of diarrhoea and mortality (9.2 and 0.3%, respectively).

In four herds (A-D), vaccinated and control animals were kept apart and in the other two (E, F), controls were mixed with vaccinated animals. In one case (see Table 4, herd F) the incidence of diarrhoea and mortality in the control group was lower than that previously recorded. However, in the other herd kept under similar conditions (Table 4, herd E) the values for the control group were similar to the mean of the last 3 years. Thus it is not possible from these results, to reach any conclusions about the effect of maintaining vaccinated animals together with, or separated from, non-vaccinated animals.

In the remaining 11 herds (Table 4, herds G-P) the incidence of diarrhoea and mortality in calves born to vaccinated animals was compared with the data of the previous 3 years. The results show again that in 10 of those herds (G-O) diarrhoea and mortality in calves from vaccinated cows (8.9 and 0.2% respectively) were far less than previously recorded in the same herds (42.3 and 5.3%, respectively).

In nine of the 17 herds involved in this study, the presence of other enteropathogens was studied the year before vaccination. In addition to rotaviruses, coronavirus and/or cryptosporidium was detected. In three herds (Table 4: LL, N and P) animals were sampled during the year of the trial and the same pathogens were found (data not shown). In this context, it should be

emphasized that, in eight of the nine herds in which other enteropathogens as well as rotavirus were present, the vaccine was also effective in decreasing morbidity and mortality due to diarrhoea.

Discussion

A growing body of evidence^{7,16} indicates that in order to guarantee adequate protection of newborn calves against enteropathogens it is necessary to supply them with continuous and sufficient amounts of neutralizing antibodies against the specific agent. This can be achieved by immunization of the dams, before delivery, with inactivated oil-adjuvanted vaccines that will induce the production and excretion of rotavirus antibodies in colostrum and post-colostrum milk^{6,16}.

In this study an oil-adjuvanted inactivated vaccine was assayed in two different experiments. The main purpose of the preliminary small-scale trial, which involved 21 pregnant cows and was conducted with the animals confined and under careful veterinary control, was to evaluate the immune response of such cows after vaccination. The efficacy of this vaccine in protecting calves from experimental challenge was also assayed in this trial.

In concordance with previous reports^{16,17}, the results of this experiment show that the level of neutralizing antibodies against homologous and heterologous rotaviruses in sera and colostrum was significantly enhanced on vaccination of the pregnant cows. Under the conditions of this trial the incidence of diarrhoea in calves born from vaccinated cows was lower than those born from unvaccinated controls. An interesting observation was the finding that all four uninoculated control calves were actively excreting rotavirus, indicating that the

natural exposure used in this experiment was a valid method of challenge. This finding is important because the dose of challenge virus used in a direct inoculation probably would not be representative of the field conditions in which spontaneous infections are continuously occurring. In this regard, difficulties (overwhelming or inadequate doses of virus) in evaluation of the efficacy of immunization by direct inoculation of calves, have been reported^{6,7}.

The results of the small experimental trial showed that the experimental vaccine was able to induce, in the milk and sera of vaccinated cows, an adequate level of neutralizing antibodies against homologous and heterologous rotaviruses; in addition, the results of the challenge were encouraging. It was therefore decided to test the vaccine in a large field trial in order to evaluate the protection afforded by the vaccine against circulating field rotavirus.

In the field trial the efficacy of the vaccine was assessed by two different approaches: by comparing the incidence of diarrhoea and mortality between vaccinated and unvaccinated groups of animals and by comparison of these data with previous values of diarrhoea and mortality (mean of the last 3 years) at the same farms.

The use of previous data to compare the results of vaccination can be problematical because of the possibility that the incidence of the various enteropathogens may change from year to year; on the other hand, when data are compared with contemporary controls that are in close contact with the vaccinated group, the incidence of diarrhoea and mortality in these controls may be underestimated because of herd immunity⁶. In order to avoid this problem, control and vaccinated groups can be separated during the trial.

The experiment shown in *Table 4* was designed to test the comparison methods. As judged by the results obtained, it was clear that the vaccine was effective in protecting calves born from vaccinated dams independently of the method used for comparison (historical or actual values of incidence of the disease). It is likely that the higher level of protection against rotavirus infection of calves born to vaccinated dams, compared with those born to control cows, was acquired through colostrum immunity.

All the herds selected for this work had experienced problems with calf diarrhoea during at least the three years before the trial. Nine of the 17 herds studied were sampled the year before the trial and, besides rotavirus, coronavirus and/or cryptosporidium were detected; in three of those herds the same enteropathogens that had been found the year before were detected again at the moment of the trial (*see Results*), suggesting that there were no significant changes in the epidemiological situation at the trial locations. This observation is relevant because the vaccine was equally effective in herds where rotavirus alone, or where rotavirus and the other two enteropathogens, were present. These results are in contradiction to those reported by Snodgrass⁶ who found that, in two herds in which rotavirus was associated with cryptosporidium, rotavirus vaccination was effective in diminishing the excretion of rotavirus but not in controlling diarrhoea. The reason(s) for such discrepancy are at present obscure and further work with a larger number of herds and animals is necessary to elucidate this problem. However, judging by the results obtained in this work, it is tempting to speculate that under the particular conditions

of cattle breeding in our country, rotaviruses could be the agents that play a major part in determining diarrhoea in newborn calves. In this regard, it is likely that biological, nutritional, environmental or management factors could influence the persistence of a given pathogen in the field, or could well influence the degree of pathogenicity of a given infectious agent or different combinations of agents⁶.

The results of this work have confirmed and extended the results of Snodgrass and colleagues⁶ regarding the effectiveness of an oil-adjuvanted inactivated rotavirus vaccine in controlling (in this case) diarrhoea in beef herds in Argentina. These data, obtained from 4066 vaccinated cattle in 17 different herds, represent the largest field trial so far for this type of vaccine.

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