Vaccination of Cows with a K99 Extract to Protect Newborn Calves Against Experimental Enterotoxic Colibacillosis

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Pregnant cows and heifers were vaccinated with a crude K99 extract prepared from an *Escherichia coli* K-12, K99 strain. A similar group, injected with an extract prepared from the K-12 parent strain, served as a control. Eleven calves were born to both groups and challenged orally with a mean of 1.6×10^{11} bacteria of enterotoxic *E. coli* B41 (O101:K99+, ST+) at the time of first colostrum uptake (1 to 7 h after birth). As a result of challenge, no death occurred in the vaccine group, but four calves died in the control group. Six calves in the vaccine group and all calves in the control group developed diarrhea. Colostral anti-O101 titers were very similar in both groups. Anti-K99 titers of colostral samples from the vaccinated dams were, however, significantly higher as compared to those of the controls. It is suggested that colostral antibodies, raised against the crude K99 extract vaccine, exerted a protective effect on newborn calves against the challenge enterotoxigenic *E. coli* O101:K99$^+$, Ent$^+$ strain.

Several workers have recently demonstrated that pili which mediate adhesion of *Escherichia coli* to the small intestine may be used as an effective vaccine against diarrhea caused by enterotoxigenic *E. coli* (ETEC) strains carrying homogenous pili (6, 8, 10, 11). Myers et al. (8) reported that a crude K99 (4) extract effectively produced antibody response in cows. However, he did not test the protective effect of this antigen. Sojka et al. (11) protected suckling lambs against experimental enteric colibacillosis by vaccinating their dams with partially purified, cell-free K99 antigen. It seemed, therefore, worthwhile to test whether or not a crude K99 extract could be succesfully used for vaccination of dams to protect their newborn calves from enterotoxic colibacillosis induced experimentally by a K99 ETEC strain.

MATERIALS AND METHODS

Crude K99 extract was prepared from *E. coli* K-12, 711, K99$^+$ (3) as described previously by Isaacson (4) without ammonium sulfate precipitation; bacteria were grown in 1-liter batches of Trypticase soy broth (TSB; BBL Microbiology Systems) in 2-liter Erlenmeyer flasks at 37°C without shaking for 20 h, sedimented by centrifugation (3,000 rpm for 30 min in 500-ml volumes), and mixed with a Biomix (Labor, Hungary) homogenizer on a 1/2 setting (ca. 10,000 rpm) for 5 min to remove K99 pili. Bacteria were sedimented again in 10-ml volumes by centrifugation as described above, and the resulting supernatant was regarded as the crude K99 extract. A similar extract was prepared from the K-12, 711 parent strain and was used as the placebo. Formaldehyde was added to the extracts to get a final concentration of 0.05%. The extracts were coded so that the persons vaccinating the dams, chal-

lenging, feeding, and observing the newborn calves did not know which material contained the crude K99 extract.

Vaccination, challenge, and observation. Twenty milliliters of the vaccine was inoculated subcutaneously two times (about 5 and 2 weeks before the expected calving data) in the flank (10 ml in both sides) of six pregnant cows and five pregnant heifers (a year-old female up to her first calving) (vaccine group). Five cows and five heifers were inoculated in the same way with the placebo (control group). Eleven calves were born to both groups (one cow in the control group had twins). Newborn calves were orally challenged with a mean of 1.6×10^{11} (range: 5.0×10^{10} to 3.0×10^{11}) bacteria of *E. coli* B41 (O101:K99$^+$, ST$^+$) in 200 ml of TSB culture (grown for 20 h at 37°C) with their first drink of colostrum. This was 1 liter of colostrum for each calf in both groups, given within 1 to 7 h after birth (average time being 2.3 h for the control group and 3.1 h for the vaccine group). Calves were fed, according to their appetite, three times a day with the dams' milk and observed for diarrhea at each feeding for 6 days. The dry matter content and coliform count of daily fecal samples from each calf were determined (1). The percentage of B41 bacteria was determined by testing 30 colonies from sheep blood agar culture of each samples for agglutinability in anti-B41 OK serum raised in rabbits (2). Based on the coliform count and percentage of B41 bacteria, the fecal B41 count was calculated. Colonies agglutinable in the anti-B41 OK serum were also tested for K99 agglutinability by using standard absorbed K99 antiserum (6) to determine the percentage of K99$^+$ B41 colonies.

Anti-O101 and anti-K99 titers of serum samples from the dams were determined before vaccination and on the day of parturition. Colostral samples were taken from the first milking. Anti-O101 titers were tested by tube agglutination by using boiled (100°C, 1

h) culture of strain B41 as antigen (2). Before the anti-K99 assays, serum and colostral whey samples were absorbed with boiled bacteria of strain B41 to remove anti-O101 antibodies. Anti-K99 titers were determined by microtiter plate agglutination, for which agitated TSB culture (20 h, 37°C) of B41 bacteria (diluted 1:2) was used. Of this antigen 0.2 ml was given to 0.1 ml of the absorbed serum dilution in 18-mm-diameter wells of plastic microtiter plates (Linbro Co.), which were gently shaken for 30 min at 37°C and read under a stereo microscope at 1:4 magnification. Titers were expressed as the reciprocal of the highest serum of whey dilution where clearly visible, loose clots of bacteria were formed.

Calves that died during the experiment were posted, and their small intestine was cultured for the presence of B41 bacteria. The fecal sample from each calf that had diarrhea was also tested for rotaviruses by using specific, fluoresceinated conjugates kindly provided by N. Zygraich (Anonyme Société) and for the presence of cryptosporidia as described previously (5, 10).

RESULTS

Differences between experimental and control groups. In the vaccine group, 6 of the 11 calves developed diarrhea and none of them died as a result of B41 challenge. In contrast, all calves developed diarrhea and four of them died in the control group (Table 1). Based on clinical, pathological, and bacteriological findings, these deaths were due to enteric colibacillosis. All dead calves had a profuse culture of B41 bacteria in the small intestine and in the feces. The fecal sample from one dead calf also contained cryptosporidia (10). These, however, were not detectable in the feces of any other calf in this experiment. Immunofluorescent microscope investigations of fecal samples from diarrheal calves were negative for calf rotavirus.

Calves from the vaccine group had lower water content in their feces compared to that of the controls (Fig. 1). Daily fecal B41 counts were very similar in both groups. Mean B41 counts per gram of dry feces was 1.0×10^{10} in the vaccine group and 2.2×10^{10} in the control group on day 1 and was 1.1×10^9 and 1.3×10^9, respectively, on day 6 of observation. From the fecal samples of vaccinated calves, an average of 57.5% of the anti-B41 serum-agglutinable colonies agglutinated in the standard absorbed anti-K99 serum on day 1 and 39.9% agglutinated on day 6. From the feces of the control calves the mean percentage of the anti-K99 agglutinable B41 colonies were 62.5% on day 1 and 52.9% on day 6. The anti-K99 titer of serum and colostral whey samples was significantly higher in the vaccine group as compared to that of the control group (Table 2).

Cows which lost their calves as a result of challenge had anti-K99 titers in their colostrum

from 0 to 1:5. Calves which had no diarrhea at all received colostrum with anti-K99 titers from 1:1 to 1:640. Anti-O101 titers of colostral samples were 1:24 in the vaccine group and 1:28 in the

TABLE 1. *Response of calves suckling vaccinated[a] or control dams to challenge with E. coli B41 (Ent+, K99+)*

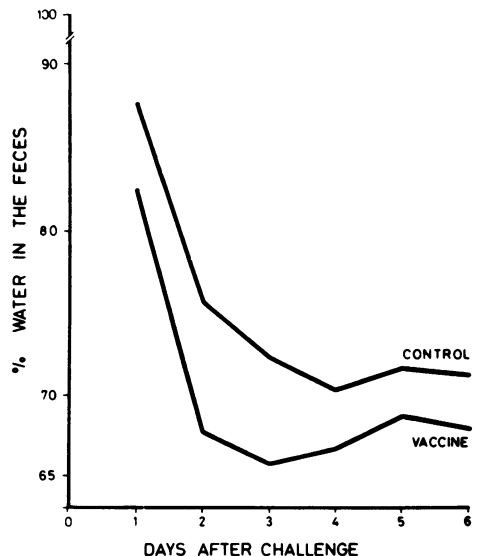| Group	Calf no.	Diarrhea after challenge			Death postinjection (h)
		Onset (h)	Severity	Duration (h)	
Vaccine	1				
	2				
	3	12	+	6	
	4	12	+	18	
	5				
	6				
	7	12	+	18	
	8	10	+	6	
	9				
	10	14	+	12	
	11	18	+	12	
Control	1	19	+	24	
	2	13	++	12	
	3	13	++	12	
	4	30	+	12	
	5	18	++	48	72
	6	17	+	12	
	7	19	+	24	
	8	18	++	12	
	9	12	++	12	24
	10	12	++	6	19
	11	9	++	18	30

[a] Vaccinated with crude K99 extract.

FIG. 1. *Fecal water content (all calves alive are included at each point) of calves born to vaccinated and control dams as a result of challenge with E. coli B41.*

TABLE 2. *Effect of vaccination with crude K99 extract on the serum and colostral antibodies of dams*

Group	Dam serum K99 titers[a]		Colostral titers[a]	
	Prevac-cination	Postvac-cination	K99	Olol
Vaccine	1.5	4.3	26.0	8.6
Control	0.0	0.0	1.2	5.0

[a] Geometric means.

control group (arithmetic mean). There were no essential differences between serum O101 titers of the two groups.

Differences between calves born from cows and heifers. Besides the above differences between experimental and control groups, there were also differences within each group when calves born from cows and those born from heifers were compared. In the control group, three calves died from heifers and one died from a cow. The three calves born to heifers died within 48 h and the one born to a cow at 4 days of age. In the vaccine group, diarrhea developed in two calves that were born to cows and in four calves that were born to heifers. The colostral anti-K99 titers of the vaccinated heifers ranged from 1:1 to 1:40 and those of the cows ranged from 1:1 to 1:640.

DISCUSSION

The difference between the vaccine and control groups was most evident in death, incidence of diarrhea, and colostral anti-K99 titers. Death occurred only in the control group and was attributed to enterotoxic colibacillosis caused by the challenge strain. In one of the calves, however, enterotoxic colibacillosis was complicated with cryptosporidiosis. Based on the profuse culture of B41 bacteria in the feces and in the small intestine of this calf, the death was attributed to the challenge organism, although it is very likely that cryptosporidia contributed essentially to its death. This was the only calf in which cryptosporidia could be detected. The incidence of diarrhea was lower and less severe in the vaccine group as compared to that of the controls. However, these differences were not reflected in the fecal counts of the challenge organism, which decreased only slightly during the 6 days of observation and were not lower in the vaccine group as compared to the controls, as could be expected from the differences in death and diarrhea. It is recognized that the fecal *E. coli* counts generally do not reflect colonization of the small intestine by ETEC (7). It could be that these calves had different degrees of colonization in the small intestine. This, however, was not tested in the present experiment.

Based on death and diarrhea, calves born to heifers seemed to be less resistant to the challenge as compared to those born to cows. In the vaccine group, the reason for these differences could be in the lower K99 titers of the colostrum of the heifers compared to that of the cows. In the control group, however, only two of the heifers and none of the cows secreted a detectable amount of anti-K99 antibodies in their colostrum. Therefore, the higher survival of the calves born to control cows in contrast to those born to control heifers could not be attributed to the anti-K99 antibodies in their colostrum.

From the four calves that died in the control group, two consumed colostrum with no detectable level of O101 antibodies, while the other two received colostrum with an anti-O101 titer of 1:40 and 1:160. Of the six control calves that seemed to be protected from death caused by the challenge organism, four had access to a colostrum which did not contain detectable level of anti-O101 antibodies. It is believed that the protection provided by the colostrum of these six cows which did not lose their calves can not be attributed to anti-O101 antibodies, but to some nonspecific effects (12).

In addition to the differences between calves born to cows and those born to heifers, there was a more striking difference related to the vaccination. The difference in K99 titers between the two groups can be explained as a result of vaccination. To our best knowledge the only difference between the vaccine and the placebo was the presence of K99 antigen on the K-12, K99 strain from which the crude extract vaccine was prepared. At the same time this antigen was absent from its parent K-12 strain. The absence of K99 antibodies in the colostrum was generally related to the diarrhea and death of the calves. Therefore, it is concluded that protection against the B41 challenge was related to the presence of K99 antibodies in the colostrum, which resulted from vaccination with the crude K99 extract.

The present study confirmed the results of Morgan et al. (7) and of Sojka et al. (12) obtained in pigs and lambs with purified or partially purified K99 and served to demonstrate that under farming conditions a protective effect can be achieved with a crude, unconcentrated K99 extract against colibacillosis induced by a K99+ ETEC strain. It seems, therefore, reasonable to use crude K99 extracts to protect newborn calves from enterotoxic colibacillosis caused by K99+ ETEC strains.

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