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Protection of Calves Against Fatal Enteric Colibacillosis by Orally Administered *Escherichia coli* K99-Specific Monoclonal Antibody

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A monoclonal antibody (MCA) to enterotoxigenic *Escherichia coli* K99 antigen agglutinated K99$^+$ enterotoxigenic *E. coli* strains B44 (O9:K30;K99;F41:H−) and B41 (O101:K99;F41:H−) grown at 37°C but not at 18°C. The MCA, which was characterized as immunoglobulin G1, reacted specifically with K99 antigen in an enzyme-linked immunosorbent assay and precipitated radiolabeled K99 antigen. A total of 45 colostrum-fed and colostrum-deprived calves were used in three separate trials to determine whether the orally administered K99-specific MCA would prevent diarrhea caused by strain B44. Twenty-eight calves were fed 1 ml of mouse ascitic fluid containing K99-specific MCA at 10 h of age and were orally challenged with strain B44 at 12 to 14 h of age. Control calves either received no placebo or were fed 1 ml of mouse ascitic fluid containing fibronectin-specific MCA at 10 h of age. There was no difference in the incidence of diarrhea between the two groups after challenge. However, the severity of diarrhea, as evaluated by the proportion of calves in each group that developed severe dehydration, the degree of clinical dehydration, the degree of clinical depression, the degree of weight loss, and the duration of diarrhea after challenge was significantly reduced in calves that received the K99-specific MCA. The mortality rate was also significantly lower ($P < 0.001$) in the treated (29%) than in the control (82%) group. These results suggest that orally administered K99-specific MCA can prevent severe fatal enteric colibacillosis.

Enterotoxigenic *Escherichia coli* (ETEC) strains can produce fatal diarrhea in neonatal calves. These organisms possess at least two known virulence factors: production of enterotoxins, which produce diarrhea by a mechanism of villous hypersecretion (21), and surface antigens, known as pili or fimbrial adhesins, which facilitate colonization of the small intestine. The K99 pilus antigen is one of the major adherence factors found on ETEC of neonatal calves (6, 9).

Since colonization is considered to be an essential step in the pathogenesis of enteric colibacillosis, it was postulated that prevention of bacterial adherence in the small intestine would reduce the severity of the disease. This could be achieved immunologically by introducing K99-specific antibody into the gut lumen of newborn calves. Acres et al. demonstrated that newborn calves challenged with ETEC are protected from fatal diarrhea if they ingest the colostrum of cows previously vaccinated with purified K99 antigen (2). Several other vaccine trials using a variety of K99-containing preparations, including whole cell bacterins and crude cellular extracts, also demonstrated that passive antibody against the K99 antigen prevents severe fatal enteric colibacillosis when ingested by newborn calves soon after birth (1, 13, 23–25). The K99 antibody was thought to prevent diarrhea by acting locally in the small intestine to prevent colonization. In all of these studies, K99 antibody was delivered to the intestines of suckling calves through the colostrum of dams which were vaccinated before parturition.

Several commercial vaccines have become available as a result of these studies and are being used with apparent success. However, there are some recognizable disadvantages to these vaccines. Pregnant cows must be handled for vaccination twice during the first year and once during each subsequent year of the vaccination program (14). Some livestock owners are reluctant to accept the cost and inconvenience of preventive vaccination unless they have recently experienced an outbreak of enteric colibacillosis in their herds. Such outbreaks are

difficult to predict because the epizootiology of neonatal calf diarrhea includes a variety of management and environmental factors as well as several different etiological agents. These concerns prompted investigation of an alternative method for direct passive immunization of newborn calves, namely, the oral administration of K99-specific, hybridoma-derived monoclonal antibody (MCA) shortly after birth.

The advent of hybridoma technology for the production of MCA has sparked intense, widespread interest in the biomedical community. Since the report by Kohler and Milstein in 1975 (19), a broad range of investigative, diagnostic, and clinical applications have been suggested for these highly specific reagents. Of particular interest to veterinary medicine are potential applications of MCA for development of specific diagnostic assays and for passive immunization against a variety of infectious diseases (20). The use of MCA to passively protect mice and sheep against bluetongue virus was recently reported (18). To our knowledge, the present study describes the first clinical use of an orally administered MCA for passive immunization of newborn domestic animals against a bacterial disease.

MATERIALS AND METHODS

MCA production. The hybridoma technology used in the preparation of the K99-specific MCA employed in these trials has been described elsewhere (8). Briefly, K99 pilus antigen, purified according to the method outlined previously (15), was provided by R. E. Isaacson (University of Michigan, Ann Arbor). The antigen (50 μg) in complete Freund adjuvant was injected subcutaneously into 9-week-old BALB/c mice. A second inoculation of antigen (30 μg) was given intravenously 7 weeks later. Six days after the intravenous dose, a third 30-μg antigen dose was administered intravenously, and 3 days later the mice were killed and the spleens were removed. Hybridomas were produced by polyethylene glycol-mediated fusion of mouse spleen cells with mouse plasmacytoma cell line P3-NS-1-Ag 4/1 (provided by L. Furcht, University of Minnesota, Minneapolis) by the method of Geftler et al. (7). The resulting hybridomas were grown in selective medium containing hypoxanthine, aminopterin, and thymidine in 24-well tissue culture plates. Supernatants from wells with hybridoma clones were assayed for K99-reactive antibody by an enzyme-linked immunosorbent assay (ELISA), using purified K99 as the antigen (30). Hybridoma monoclones producing large amounts of K99-specific MCA were isolated by limiting dilution.

A single clone (2BD4E4) which produced large amounts of K99-specific antibody as detected by ELISA was selected and injected intraperitoneally into specific-pathogen-free BALB/c mice, which were preconditioned with pristane (2,6,10,14-tetramethylpentadecane; Aldrich Chemical Co., Milwaukee, Wis.) for production of MCA. At 10 to 60 days after intraperitoneal injection, mice were killed, and ascitic fluid containing the K99 MCA was aspirated, clarified by centrifugation at 1,400 × *g* for 10 min, and stored at −20°C.

Characterization of MCA. Characterization of MCA was performed by several techniques, including ELISA, bacterial agglutination, and immunoprecipitation. The ELISA was performed by utilizing microtiter plates sensitized with purified K99 antigen according to standard methods (30) and rabbit isotype-specific agents (Litton Bionetics, Kensington, Md.). Bacterial agglutination was performed with strains of *E. coli* grown overnight on Minca agar containing 1% IsoVitaleX (Minca-Is) (10) at 37 or 18°C.

The specificity of the MCA was further demonstrated by immunoprecipitation of radiolabeled ETEC strain B44. ETEC was grown at 37°C in 1 ml of Minca-Is broth. After overnight growth, 0.05 ml of ETEC was transferred into 1 ml of fresh Minca-Is. After incubation for 1 h at 37°C, the Minca-Is was supplemented with 100 μCi of L-[^{35}S]methionine (1,210 Ci/mmol; Amersham Corp., Arlington Heights, Ill.), and incubation was continued for 0.5 h. ETEC was pelleted and then disrupted at 4°C by a 10-min treatment with 0.5 ml of lysozyme at 10 mg/ml in TE-10 (0.01 M Tris-hydrochloride, 0.001 M EDTA, pH 7.2), a 10-min treatment with 0.5 ml of modified RIPA (0.02 M Tris-hydrochloride, 0.3 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate [SDS], pH 7.2), and pulse sonication for 30 s (Sonifier Cell Disruptor 350, Branson Sonic Power Co., Danburg, Conn.). Unlysed cells and cell debris were removed by adding 0.4 ml of chicken egg albumin at 1 mg/ml in buffer C (0.01 M Tris-hydrochloride, 0.15 M NaCl, 0.001 M EDTA, 0.05% Nonidet P-40, pH 7.2) and centifuging at 15,600 × *g* for 10 min at 4°C (Eppendorf centrifuge 5414).

The K99 antigen present in the clarified ETEC lysate was identified by utilizing Formalin-fixed *Staphylococcus aureus* Cowan strain I (16) to precipitate the antigen-MCA complexes. All reactions were carried out at 4°C, and all centrifugations were at 3,000 × *g* for 5 min at 4°C (Beckman TJ-6R tabletop centrifuge, TA-24 rotor). Nonspecific binding was reduced by reacting 0.2 ml of the lysate with 0.002 ml of fibronectin-specific MCA in 0.8 ml of RIPA buffer (0.01 M Tris-hydrochloride, 0.15 M NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, pH 7.2) (3) for 15 min. This was followed by a 15-min incubation with 0.005 ml of rabbit anti-mouse immunoglobulin (Litton Bionetics). The resulting nonspecific antibody-antigen complexes were precipitated by addition of 0.1 ml of a 10% (vol/vol) *S. aureus* suspension for 10 min, followed by centrifugation. The supernatant was reacted with 0.002 ml of the K99 MCA, and the antigen-antibody complexes were precipitated as described above. The antigen-antibody-*S. aureus* complex was washed three times in 1 ml of RIPA buffer and dissociated for SDS-polyacrylamide gel electrophoresis by boiling for 2 min in sample buffer (17). SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli, and radiolabeled proteins were detected on Kodak XAR-5 film at −70°C by fluorography (4, 17).

PHA. For passive hemagglutination (PHA) turkey erythrocytes were fixed with formaldehyde by the method of Sequeira and Eldridge (28), treated with tannic acid, and sensitized with an optimal dilution of

purified K99 antigen preparation. Test sera were titrated by fourfold dilutions for an initial dilution of 1:20 in V-well microtiter plates, using 0.1 M phosphate-buffered saline (pH 7.2) containing 1% heat-inactivated normal turkey serum. A 25-μl portion of a 1% suspension of sensitized turkey erythrocytes in phosphate-buffered saline containing 1% heat-inactivated normal rabbit serum was added to each well. After gentle agitation, the plates were covered and allowed to stand at room temperature for 30 min before agglutination titers were recorded. Standardization of the sensitivity and specificity of the PHA system will be reported in detail elsewhere.

Clinical evaluation of K99 MCA. Three separate challenge trials were conducted; trial 1 was at the Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada, and trials 2 and 3 were at the University of Minnesota, St. Paul. Certain features of the experimental design were common to all three trials. Newborn dairy calves were purchased at birth from commercial farms within a 60-mile radius of the research facilities and maintained in individual isolation rooms. Test calves received 1 ml of mouse ascitic fluid containing the K99-specific MCA in 1 liter of skim milk in a nursing bottle at 10 h of age. Ascitic fluid used in all three trials contained the same K99 antigen-binding activity, as determined by endpoint titration in ELISA. By using the PHA system, the anti-K99 titer of the MCA contained in each dose of ascitic fluid was determined to be 1:12,000. All calves were subsequently challenged at 12 to 14 h of age with ETEC strain B44 (O9:K30;K99;F41:H−) in 1 liter of skim milk. Calves were fed twice a day and clinically examined daily after challenge. The clinical status of calves was evaluated on the basis of fecal consistency, degree of clinical dehydration, and degree of clinical depression, as follows. For fecal consistency, 0 = normal, i.e., manure firm and well formed; 1 = abnormal feces but not diarrheic, i.e., manure slightly softer than normal; 2 = mild diarrhea; 3 = severe watery diarrhea. For degree of clinical dehydration, 0 = normal, i.e., eyes bright and skin pliable; 1 = mild dehydration; 2 = severe dehydration (>10% of body weight). For degree of clinical depression, 0 = normal; 1 = mild depression; 2 = moderate depression; 3 = severe depression, i.e., calf unable to stand or nurse. A maximum clinical score, which ranged from normal (0) to death (8), was calculated by adding the three scores for the clinical evaluations just described (1).

Minor differences in protocol among the clinical trials are described below.

(i) Trial 1. Fourteen colostrum-fed calves which had been allowed to nurse normally were used. Every second calf received K99-specific MCA, while the alternate calves received skim milk only (no placebo). All calves were challenged with an inoculum of ETEC strain B44 containing an average of 2.6×10^{10} viable organisms. The inoculum was grown on Minca-Is as previously described (1), washed off the plates with sterile phosphate-buffered saline, diluted to a concentration of 1.5×10^{10} viable cells per ml, and frozen at −70°C in 4-ml aliquots. Control calves were bled at the time of challenge and 12 h after challenge. Treated calves were bled at the time the K99-specific MCA was fed (10 h) and again 12 h postchallenge. Serum was stored frozen and later examined for the presence of K99 antibody by PHA.

(ii) Trial 2. Nine colostrum-deprived calves were used. These calves were removed immediately from their dams at birth. Four received a placebo, and five received K99-specific MCA at the discretion of the principal investigator. The placebo consisted of 1 ml of mouse ascitic fluid containing fibronectin-specific MCA. All calves were challenged with an average ETEC strain B44 inoculum of 5×10^9 viable organisms from a fresh 4-h Minca-Is broth culture initiated when each calf was 8 h old. Dosage was adjusted by dilution with sterile broth and measurement of optical density in a Spectronic 20 spectrophotometer at 420-nm wavelength. A 10-ml sample of the adjusted broth culture was used for challenge. Any calf that died during the clinical evaluation period was necropsied.

(iii) Trial 3. Twenty-two colostrum-deprived calves were used. These calves were removed immediately from their dams at birth. Six received a placebo, and sixteen received K99-specific MCA in a blind study. This trial differed from trial 2 only in that the principal investigator did not know which calves received treatment. In trials 2 and 3, calves were weighed daily.

RESULTS

Characterization of clone 2BD4E4 MCA. The MCA from clone 2BD4E4 was shown to be immunoglobulin G1 by ELISA with rabbit anti-mouse immunoglobulin G1 antibody. A pool of ascitic fluid from this clone exhibited a titer of 10^{-5} in the K99-ELISA system, using techniques similar to those described previously (30). Immunoglobulin constituted 45 to 50% of the protein in the ascites fluid as determined by SDS-polyacrylamide gel electrophoresis. The antibody was found to be reactive only with K99 antigen in radioimmunoprecipitation of L-[^{35}S]methionine-labeled *E. coli* lysates followed by SDS-polyacrylamide gel electrophoresis (Fig. 1). This immunoglobulin at an initial concentration of 10 mg/ml agglutinated ETEC strains B44 and B41 grown at 37°C (K99 pilus expressed) to a dilution of 10^{-4}, but did not agglutinate cells grown at 18°C (K99 pilus not expressed) even when used at 10^{-1}.

Clinical response of calves after challenge. Treatment with the K99-specific MCA did not affect the incidence of diarrhea after challenge with ETEC strain B44. There was no difference in the proportion of treated and control calves that became diarrheic in any of the three trials as judged by occurrence of a fecal consistency score of 3 (Table 1), nor was there a significant difference in the average score of fecal consistency between treated and control groups in any of the three trials (Table 2). As calculated in trial 1 only, there was also no significant difference in the time of onset of diarrhea after challenge between the control and treated calves (Table 2).

In contrast to the above-described results, there was a significant difference in the incidence of severe dehydration between control and treated calves. The proportion of calves that

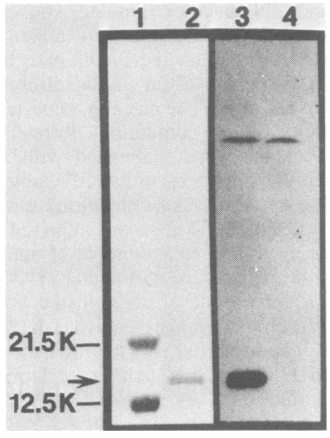

FIG. 1. Immunoprecipitation of an L-[^{35}S]methionine-labeled lysate of ETEC strain B44 with the K99-MCA orally administered to calves. Immunoprecipitates were subjected to electrophoresis in a 12.5% acrylamide slab gel. Proteins in lanes 1 and 2 were detected by Coomassie blue staining before salicylation of the gel. Molecular weight markers (in thousands [K]) are indicated in lane 1. The K99 antigen used subsequently for production of K99-MCA is indicated by the arrow in lane 2. Lane 3 represents the radioactive antigen precipitated from ETEC strain B44 by K99-MCA. Lane 4 represents a similar precipitate by fibronectin-specific MCA.

became severely dehydrated (score = 2) after challenge was significantly lower in calves receiving the K99-specific MCA as compared with control calves in all three trials (Table 1). This reduction was highly significant ($P < 0.001$) when the results of the three trials were combined. There was also a significant reduction in the severity of systemic illness between treatment and control groups in all three trials as measured by comparison of mean scores earned for degree of clinical dehydration, degree of clinical depression, and maximum clinical score (Table 2). In addition, a significant difference was noted in the duration of diarrhea after challenge when measured in trial 1, and a significant difference was observed in the degree of weight loss between treated and control calves when measured in trials 2 and 3 (Table 2).

Treatment with the K99-specific MCA also significantly reduced mortality in challenged calves in all three trials (Table 1). Since the three trials were carried out with some variation of protocol and location, it was of interest to test the relationship of calf survival to both treatment effect and trial number. A three-dimensional analysis of mortality data from the three trials was performed by using log linear regresion. The three variables tested were administration of K99-specific MCA, calf survival, and trial number. A model which showed calf survival to be dependent on MCA administration and independent of trial number gave the best fit ($\chi^2 = 5.19$, 6 degrees of freedom). When the results of all three trials are considered together, the mortality rate in the calves receiving MCA was only 29% as compared with 82% in the control calves. This difference was highly significant ($P < 0.001$).

Sera taken from some calves in trial 1 and examined for the presence of K99 antibody by PHA showed that some K99-specific MCA was absorbed from the gastrointestinal tract into the blood. Before challenge, calves in both treatment groups had K99 serum antibody titers of less than 20. Twelve hours after challenge, calves receiving K99 MCA had K99 serum antibody titers ranging from 20 to 1,280. Calves in the control group had titers which remained at less than 20. Titers are reported as the reciprocal of the highest dilution of serum which contained K99 antibody as detected by PHA.

DISCUSSION

Treatment of calves orally with 1 ml of mouse ascitic fluid containing K99-specific MCA re-

TABLE 1. Clinical response of calves after challenge with ETEC strain B44

Trial	K99 MCA-treated calves			Controls		
	Diarrhea[a]	Clinical dehydration[b]	Death	Diarrhea	Clinical dehydration	Death
1	4/7	1/7[c]	1/7[c]	5/7	5/7	5/7
2	5/5	1/5[c]	1/5[c]	4/4	4/4	4/4
3	12/16	6/16[c]	6/16[c]	5/6	5/6	5/6
Total	21/28 (75%)	8/28[d] (29%)	8/28[d] (29%)	14/17 (82%)	14/17 (82%)	14/17 (82%)

[a] Expressed as the proportion of calves in the group that developed a fecal consistency score of 3 after challenge. See text for details of classification.

[b] Expressed as the proportion of calves in the group that developed a clinical dehydration score of 2. See text for details of classification.

[c] $P < 0.05$ versus control by one-tailed chi-square test (26).

[d] $P < 0.001$ versus control by one-tailed chi-square test (26).

TABLE 2. Mean clinical scores of calves after challenge with ETEC strain B44[a]

Trial	Calves	MCS (0 to 8)	DEP (0 to 3)	DEHY (0 to 2)	FC (0 to 3)	Wt loss (kg)	Duration of diarrhea (h)	Onset of diarrhea after challenge (h)
1	T	2.9 ± 2.7 (<0.05)	0.4 ± 1.1 (<0.025)	0.4 ± 0.8 (<0.0125)	2.0 ± 1.4 (NS)	ND	22.5 ± 3.0 (<0.025)	31.0 ± 24.9 (NS)
	C	6.1 ± 3.3	2.1 ± 1.5	1.6 ± 0.8	2.4 ± 1.1	ND	58.8 ± 42.5	14.8 ± 3.7
2	T	4.2 ± 2.2 (<0.005)	0.8 ± 1.3 (<0.005)	0.4 ± 0.9 (<0.005)	3.0 ± 0 (NS)	3.2 ± 2.8 (<0.025)	ND	ND
	C	8.0 ± 0	3.0 ± 0	2.0 ± 0	3.0 ± 0	7.6 ± 2.3	ND	ND
3	T	4.7 ± 2.7 (<0.0125)	1.3 ± 1.2 (<0.0025)	0.9 ± 1.0 (<0.0025)	2.6 ± 0.9 (NS)	3.1 ± 3.0 (<0.05)	ND	ND
	C	7.3 ± 1.6	2.7 ± 0.8	1.8 ± 0.4	2.8 ± 0.4	5.9 ± 3.3	ND	ND
Combined	T	4.1 ± 2.4 (<0.001)	1.0 ± 1.2 (<0.001)	0.7 ± 0.9 (<0.001)	2.5 ± 1.0 (NS)	3.1 ± 2.9 (<0.0025)	ND	ND
	C	7.0 ± 3.8	2.5 ± 1.1	1.8 ± 0.6	2.7 ± 0.8	6.6 ± 3.0	ND	ND

[a] All data are expressed as mean ± standard deviation; numbers within parentheses are *P* values versus control, using the one-tailed *t*-test. Abbreviations: T, treated group; C, control group; MCS, maximum clinical score; DEP, degree of depression; DEHY, degree of dehydration; FC, fecal consistency; NS, not significant; ND, not done. See test for explanation of clinical scoring.

duced the severity of diarrhea and the mortality rate after challenge with ETEC strain B44. This supports an earlier report that immunization of cows with purified K99 antigen before calving stimulated their production of K99-specific antibodies, which were passively transferred to their calves and which prevented fatal diarrhea (2).

Colonization of the small intestine, which is a necessary step in the pathogenesis of diarrhea, is a complex process that requires the ingested bacteria to survive and multiply in the intestinal lumen, contact the mucosa, and interact with the surface of the brush border and its protective layers through a series of specific and nonspecific reactions. The roles of various antigens of ETEC in this process have not been clearly defined; however, maximum colonization occurs with strains which possess both pili and capsule (5, 11, 12), suggesting that both types of antigens are important. In this study, antibody directed against only the K99 pilus of ETEC significantly reduced the severity of diarrhea caused by strain B44, which carries two attachment pili (K99 and F41) as well as the K30 capsular antigen. This suggests that the degree of colonization, and hence the clinical severity of disease, was reduced by K99-specific MCA. Since calves in trials 2 and 3 received no colostrum, it can be assumed that naturally occurring maternal antibodies to capsular and pilus antigens played no role in modulating the severity of disease in this study.

There are several possible explanations for the occurrence of transient watery diarrhea in animals which received the K99-specific MCA. First, the dose of MCA administered might not have completely blocked colonization. Second, even though K99-mediated attachment was prevented, sufficient F41-mediated adherence might have occurred to cause transient diarrhea (22). Third, the number of bacteria in the challenge inoculum might have been large enough to produce sufficient enterotoxin to stimulate a hypersecretory diarrhea even in the absence of bacterial attachment to the villous surface (1).

The fact that 29% of calves receiving the K99-specific MCA died may be due to several aspects of the experimental protocol designed to rigorously test the protective value of the K99-specific MCA. Many elements of the clinical trials did not favor calf survival. Most importantly, calves in trials 2 and 3 were colostrum deprived, which may have contributed to the development of septicemia or other concurrent infections. In fact, postmortem examination of protected calves dying in these trials revealed the presence in some calves of enteric rotavirus and coronavirus infections. These concurrent infections undoubtedly contributed to the severity of dehydration and subsequent death. In all of these trials calves received no treatment for diarrhea or dehydration. Fluid intake was restricted to twice-daily feedings, and skim milk was fed in place of whole milk or milk replacer. Despite these obstacles, 71% of calves receiving K99-specific MCA survived challenge.

Since calves appear most susceptible to ETEC infection during the first day of life (27), a protocol of treatment and challenge within the first 12 h of age was chosen to rigorously test the effectiveness of oral MCA administration for protection against enteric colibacillosis. It would be of interest in future studies to manipulate the temporal relationship of treatment and challenge during the first few days of life to further define the limits of protection possible with orally

administered K99-specific MCA (29). Another objective of future studies should be to evaluate the significance of adsorption of mouse immunoglobulin G1 MCA from the intestinal lumen of the calf. In this study, none of the calves used demonstrated apparent untoward effects as a result of MCA administration. However, it would be of interest to follow calves through a regimen of repeated treatments with K99-specific or other mouse MCA to evaluate possible reactions to these foreign immunoglobulins.

The occurrence of enteric colibacillosis in calves is often unpredictable and sporadic and is influenced by the complex interaction of a variety of factors, such as colostrum ingestion, weather changes, and stocking rates. Since the incidence of disease is low in some years, it may be hard to convince livestock producers that vaccination of cows with bacterins for the prevention of neonatal diarrhea is cost effective. If outbreaks of enteric colibacillosis subsequently occur, it is then too late to vaccinate cows since development of passive protection takes a minimum of 2 weeks (1). The oral administration of K99-specific MCA to calves during the first 12 h of age may be an effective way to reduce the economic loss to cattle owners when outbreaks of enteric colibacillosis caused by K99$^+$ ETEC occur in unvaccinated herds.

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