The Role of K Antigens of Enteropathogenic *Escherichia coli* in Colonization of the Small Intestine of Calves

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ABSTRACT

The colonizing and proliferating abilities of enterotoxigenic acapsular or K99⁻ mutants of bovine enteropathogenic *Escherichia coli* strains were compared with those of their capsulated and K99⁺ parent strains in the small intestine of infected colostrum-fed calves. Calves infected with the enteropathogenic *E. coli* parent strains developed profuse diarrhea and severe dehydration. None of the calves which received the acapsular mutant developed diarrhea and one of three calves inoculated with the K99⁻ enterotoxigenic mutant developed moderate diarrhea. The parent enteropathogenic *E. coli* strains colonized the middle and lower small intestine: in these areas a layer of specific immunofluorescence against the enteropathogenic *E. coli* covered most villi and 80% of the organisms were associated with the intestinal wall. The acapsular mutant strain failed to colonize the small intestine and fluorescent bacteria were not observed in any area of the small intestine. The K99⁻ mutant proliferated to a lesser extent than did the K99⁺ parent strain in all areas of the small intestine but moderately colonized the lower small intestine where fluorescent bacteria were observed to cover parts of the intestinal villi.

RÉSUMÉ

Cette expérience consistait à vérifier si des mutants entérotoxinogènes, dépourvus de capsule ou de l'antigène K99 et tissus de souches entéropathogènes bovines d'*Escherichia coli*, pouvaient former des colonies et proliférer dans l'intestin grêle de veaux expérimentaux qui avaient bu le colostrum maternel; elle visait aussi à comparer ces aptitudes avec celles des souches dont ils originaient, lesquelles possédaient une capsule et l'antigène K99. Les veaux auxquels on administra les souches parentales développèrent une diarrhée profuse et une déshydratation marquée. Par ailleurs, aucun de ceux qui reçurent le mutant dépourvu de capsule ne développa de diarrhée, tandis qu'un des trois auxquels on avait administré le mutant entérotoxinogène dépourvu de l'antigène K99 développa une diarrhée modérée. Les souches parentales formèrent des colonies dans le jéjunum et l'iléon; dans ces segments, une couche d'immunofluorescence spécifique à l'endroit des souches en cause recouvrait la plupart des villosités et 80% des colibacilles adhéraient à la muqueuse intestinale. Le mutant dépourvu de capsule ne réussit pas à former des colonies dans l'intestin grêle et on n'y retrouva aucune bactérie fluorescente. Le mutant dépourvu de l'antigène K99 proliféra un peu moins que ne le fit sa souche mère, dans tous les segments de l'intestin grêle; il réussit cependant à proliférer, dans une certaine mesure, dans la demi distale de l'intestin grêle et on y détecta des bactéries fluorescentes qui recouvraient partiellement certaines villosités.

INTRODUCTION

In enterotoxic diarrheal diseases caused by *Escherichia coli* in calves and other animal species, intestinal colonization plays a key role in the pathogenesis of the disease (1,5,8,12,13). Adhesion of *E. coli* to the intestinal epithelium enables the organism to overcome the cleansing action of peristaltic motility of the gut and to reach large numbers in the small intestine where they produce and deliver effective levels of enterotoxin in close proximity to the target enterocytes (17,18,20).

Among enteropathogenic *E. coli* (EPEC) isolated from calves and lambs, the K99 pilus is clearly implicated as a mediator of adhesion to the small intestine (3,8,9, 14,19). The capsular polysaccharide K antigen of some bovine EPEC strains has also been ascribed a role in the adhesion of *E. coli* to the small intestinal epithelium (19), but its significance is not clear.

The present work was undertaken to investigate the role of the capsular polysaccharide and the K99 pili in adherence and multiplication of bovine EPEC in the small intestine of colostrum-fed calves.

MATERIALS AND METHODS

E. COLI STRAINS

An EPEC strain 505 (0101:K28, K99) was obtained from Dr. L.L.

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Myers of the Veterinary Research Laboratory, Bozeman, Montana. A spontaneously occurring noncapsulated mutant (K^-) of strain 505 was selected from colonies of the parent strain grown on MacConkey agar. Its K^- status was confirmed by demonstrating its inagglutinability in homologous K antiserum, and its agglutination in homologous O antiserum. Another EPEC strain that was used was B44 (O9:K30,K99); its $K99^-$ derivative was obtained by removal of the K99 plasmid by treatment with acridine orange (11). Both parent and $K99^-$ derivative were shown to have similar growth rates *in vitro*. Both EPEC strains and their mutants were shown to produce enterotoxin detectable by the infant mouse assay of broth culture supernatants (4). The *E. coli* K12 strains 711 and 711(K99), a derivative that carried the K99 plasmid from the bovine EPEC strain B41 ($O101:K^-$,K99), were used for preparation of K99 antiserum. All *E. coli* strains were chromosomal mutants resistant to nalidixic acid.

PRODUCTION OF ANTISERA

OK antisera against *E. coli* strains 505, B44, and 711(K99) were produced by repeated intravenous injections of live cultures in rabbits (21). The antiserum against *E. coli* strain *711*(K99) was absorbed with live culture of the parent strain, 711. The absorbed antiserum did not agglutinate the parent strain but agglutinated *E. coli* 711(K99) and was used as monospecific for the K99 antigen. O antiserum against *E. coli* strain 505 was produced in rabbits by repeated intravenous injections of boiled cultures (21).

EXPERIMENTAL CALVES

Fourteen eight to 14 hour old colostrum-fed Holstein Friesian bull calves were obtained from local farms and kept in individual pens. The calves were starved for eight hours prior to oral inoculation at 16 to 22 hours of age with 10^{11} *E. coli* grown on Minca (3) plates at 37°C for 12 hours. The calves were fed the inoculum in 500 mL milk replacer through a nipple attached to a bottle. Four calves were infected with *E. coli* strain 505, four with the *E. coli* $505K^-$ mutant, three with *E. coli* B44 and three with the $K99^-$ form of *E. coli* B44. After inoculation, the calves were observed at one hour intervals for evidence of diarrhea.

ENUMERATION OF *E. COLI* IN THE INTESTINE OF CALVES

The calves were euthanized 16 hours postinoculation or earlier in those cases where very severe diarrhea and dehydration developed. The small intestine was carefully unfolded and tied at several positions along its length to ensure that the contents remained in position. The entire small intestine was removed from the calves and divided into three approximately equal parts. The contents of each part of the small intestine were removed, measured and placed in separate beakers in a refrigerator. Each part of the small intestine was further divided into four segments of equal lengths and each segment was flushed gently with 50 mL of cold (4°C) sterile saline solution by inverting five times. The washings from the four segments were added to the contents and refrigerated. Each segment of intestine was everted on a glass rod and the mucosa was removed by drawing the segment through the partially closed jaws of a staple remover. The mucosal scrapings were refrigerated. After all six samples (wall scrapings and contents of each part of the intestine) were prepared, each was homogenized in a chilled Waring blender. Serial tenfold dilutions of each sample were made with cold sterile saline solution and 0.1 mL volumes of three dilutions were plated on MacConkey agar containing nalidixic acid (50 μg/mL). Following incubation of the agar plates, the identity of the colonies was confirmed for at least five colonies per plate by slide agglutination in homologous OK antiserum.

INDIRECT FLUORESCENT ANTIBODY TECHNIQUE

Sections 6 μm thick were cut from frozen segments of anterior, middle and lower small intestine in a Cryostat at -20°C. The segments representing the anterior, middle and lower small intestine had been taken from the centre of the anterior, middle and posterior one thirds, respectively. Two additional sections of intestine were sampled, one 5 cm from the pylorus and the other 5 cm from the ilcocecal valve. The sections were mounted on glass slides, fixed in ethanol and reacted with either homologous OK antiserum at a dilution of 1/160 or undiluted K99 antiserum. The slides were held in a moist chamber for 30 minutes at 22°C after which they were washed for 30 minutes with three changes of phosphate-buffered saline (PBS) (0.01 M pH 7.2). The sections were air-dried and stained with guinea pig antirabbit globulin conjugated with fluorescein (4), washed again in PBS, air-dried and examined with a Zeiss Epi fluorescent microscope. Control slides were prepared using normal rabbit serum in the first step.

RESULTS

CLINICAL RESPONSES AND POSTMORTEM OBSERVATIONS

Table I summarizes the clinical responses and postmortem obser-

TABLE I. Clinical and Postmortem Features of Calves Following Oral Inoculation with Selected Pairs of *E. coli* Strains

		Calves[a]		
Strain	*E. coli Serogroup*	Diarrhea	Died[b] or Killed Before 16 h Postinoculation	Hyperaemic Ileal Mucosa
505	0101:K28,K99	4/4	1/4[b]	4/4
$505K^-$	0101:K28$^-$K99	0/4	0/4	0/4
B44	09 :K30,K99	3/3	2/3	3/3
$B44K99^-$	09 :K30,K99$^-$	1/3	0/3	0/3

[a]Number of calves affected/number challenged
[b]Enumeration of the *E. coli* in the intestine of this calf started within ten minutes of death

vations in the four groups of calves. Of the four calves infected with the EPEC strain 505 three developed severe diarrhea with marked dehydration while the fourth developed mild to moderate diarrhea. The diarrhea started three to five hours postinoculation (PI) and the calves refused to drink milk at the second feeding, 8 h PI. The intestinal contents were watery and yellowish green, and the intestine was hyperaemic especially in the lower areas where petechial haemorrhages were evident on the mucosal surface. One of these calves died 14 h PI. Enumeration of the *E. coli* in the intestine of this calf started within ten minutes of its death.

All four calves infected with the acapsular mutant (K^-) failed to develop diarrhea and remained healthy throughout the observation period. They were hungry and drank all their milk in the second feeding. The small intestine had a normal appearance and contained yellowish fluid.

Two of the three calves infected with *E. coli* strain B44 developed very severe diarrhea 6 h PI and had to be euthanized 12 to 14 h PI. The third calf developed moderate diarrhea which started 9 h PI. The feces of the two calves which were killed before the scheduled end of the experiment were pinkish in colour; their small intestines were hyperaemic in the lower half with marked patchy haemorrhages on the mucosal surface and with watery, pinkish brown contents. The large intestine contained a considerable amount of pinkish watery fluid. The third calf had yellowish watery feces and clear yellowish intestinal contents.

One of the three calves infected with the $K99^-$ form of B44 developed moderate diarrhea which started 9 h PI and had watery intestinal contents. The remaining two calves did not develop diarrhea and remained healthy and alert throughout the observation period. The intestinal contents were semifluid in consistency and of yellowish green colour. The intestinal appearance was normal.

THE NUMBERS AND DISTRIBUTION OF *E. COLI* IN THE SMALL INTESTINE

Figure 1 shows the total numbers of viable *E. coli* recovered from the wall scrapings and intestinal contents in each of the three areas of the small intestine for each of the four groups of calves. Each bar represents the mean of the total viable *E. coli* for all the calves in a group. The complete data for all the calves are presented in Tables II and III.

In calves infected with *E. coli* strain 505 the total viable counts were in the range of 10^8 to 10^{10} in the anterior small intestine, 10^9-10^{11} in the middle small intestine and 10^{10}-10^{11} in the lower small intestine (Table II). In the anterior small intestine there were greater numbers of *E. coli* in the contents than in the wall preparations in all calves except one calf (#3, Table II) which died 14 h PI. In the middle and lower small intestine there were approximately ten times as many *E. coli* 505 associated with the wall as with the lumen in all calves.

In the case of calves infected with the acapsular mutant of *E. coli* 505 there were significantly fewer *E. coli* in all areas of the small intestine compared with the numbers for the calves infected with the capsulated strain 505. The numbers were, on the average about 10^3 lower than those in calves infected with the parent strain. The numbers increased slightly from anterior to posterior and there were always more *E. coli* in the lumen preparations than in the wall preparations (Fig. 1, Table II).

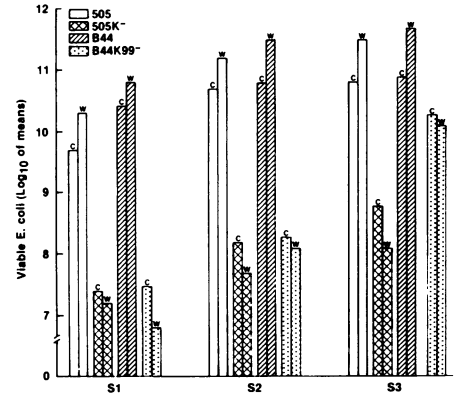

Fig. 1. Total number of viable *E. coli* (log_{10} of means) recovered from the lumen contents (C) or the wall scrapings (W) of washed intestinal segments of calves exposed to selected EPEC strains. Four calves were infected with the EPEC strain 505, four with the noncapsular mutant (K^-) of strain 505, three with the EPEC strain B44 and three with the $K99^-$ form of *E. coli* B44. S1 = anterior small intestine, S2 = middle small intestine, S3 = posterior small intestine.

The pattern of colonization of the small intestine by *E. coli* strain B44 was similar to that for strain 505. The calves infected with the $K99^-$ form of B44 had markedly fewer *E. coli* in all areas of the small intestine than those infected with the parent strain. In the anterior and middle small intestine the difference was about 1000-fold while in the posterior small intestine it was only about tenfold (Fig. 1).

Figure 2 shows the mean proportion of total viable *E. coli* associated with the wall preparations. A high proportion of the total *E. coli* was recovered from the wall

TABLE II. Viable *E. coli* in Wall Scraping and Contents of the Small Intestine of Calves Given an Oral Dose of 10^{11} Enteropathogenic *E. coli* Strain 505 or its Acapsular Mutant

Total *E. coli* ($x10^9$)	Calves given *E. coli* 505				Calves given the acapsular mutant			
	1	2	3	4	1	2	3	4
Anterior[a]								
Wall	0.5	0.04	81	0.5	0.0004	0.07	0.0003	0.0003
Contents	3.9	0.3	33	0.3	0.0002	0.09	0.0006	0.0002
Middle[a]								
Wall	113	7	354	200	0.005	0.2	0.01	0.001
Contents	60	6	74	51	0.005	0.3	0.4	0.06
Posterior[a]								
Wall	242	110	700	192	0.2	1.4	0.06	0.3
Contents	64	54	43	84	0.5	0.06	0.1	0.3

[a]Anterior, middle and posterior areas of the small intestine

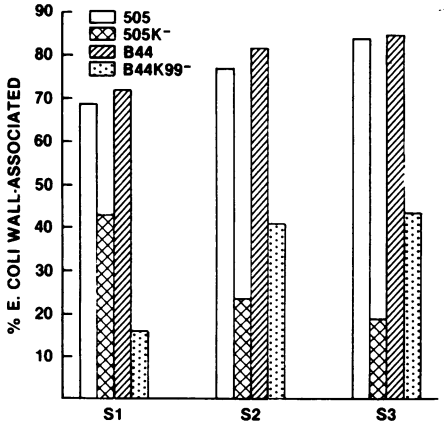

Fig. 2. Percentage of the total viable *E. coli* recovered from the intestinal wall of washed intestinal segments of calves exposed to selected EPEC strains. Each bar represents the mean for the calves in the group. S1 = anterior small intestine, S2 = middle small intestine, S3 = posterior small intestine.

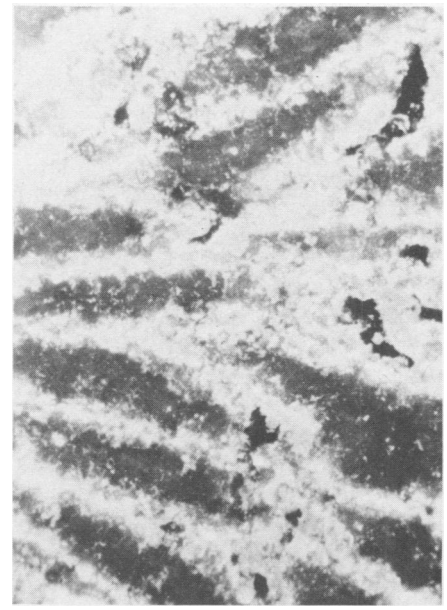

Fig. 3. Intestinal villi covered by fluorescent organisms of the enteropathogenic *E. coli* strain 505. Layers of *E. coli* strain 505 adhered to the epithelium along the entire length of the villi. Frozen section of the lower small intestine was stained by indirect immunofluorescence, using OK antiserum against *E. coli* 505 and FITC-labelled guinea pig anti-rabbit gammaglobulin. Final magnification is 50.

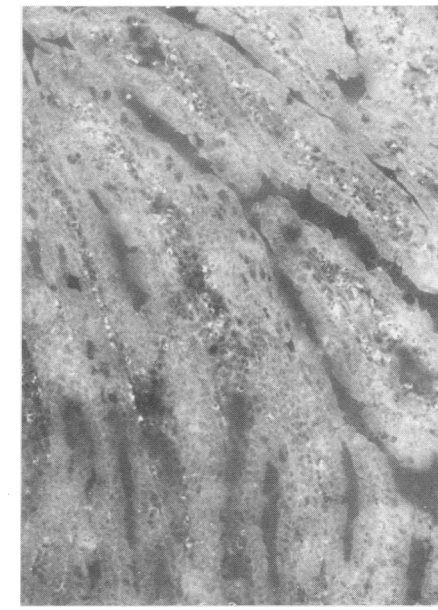

Fig. 4. Demonstration of the failure of the acapsular mutant (K^-) of the enteropathogenic *E. coli* strain 505 to adhere to the intestinal epithelium of an infected calf. Frozen section of the lower small intestine stained by indirect immunofluorescence using O antiserum against the *E. coli* 505 and FITC-labelled guinea pig anti-rabbit gammaglobulin. Final magnification is 50.

preparations in all the areas of the small intestine in calves infected with the *E. coli* strains 505 and B44 (Fig. 2, Table II and III). Usually the percentages of the organism that were wall-associated increased from anterior to middle to posterior segments. In the calves infected with the acapsular mutant of *E. coli* 505 or the K99⁻ form of *E. coli* B44, low percentages of *E. coli* were recovered from the wall preparations in all areas of the small intestine. The acapsular mutant of strain 505 showed a marked decrease in the percentages of wall-associated organisms from the anterior to posterior segments whereas the K99⁻ form of B44 showed a reverse relationship with marked increase in the percentages of wall-associated organisms from anterior to posterior segments.

FLUORESCENT ANTIBODY STUDIES (FA)

All calves infected with *E. coli* strain 505 had a layer of specific immunofluorescence which covered the majority of the villi from the tip to the base in the middle and lower small intestine (Fig. 3). However, less immunofluorescence was seen in sections from the middle small intestine than in sections from the lower small intestine. No fluorescing bacteria were seen in sections from the anterior small intestine in any calf. Fluorescent bacteria were not observed in any section from any area of the small intestine in the calves infected with the acapsular mutant of strain 505 (Fig. 4).

In calves infected with the parent *E. coli* strain B44, layers of fluorescent bacteria were observed adherent to most villi in all intestinal areas except close to the pylorus. In the middle and lower small intestine a fluorescing layer covered the entire epithelial surface of the villi. In the anterior small intestine fluorescent bacteria were observed in only some areas and appeared to be confined to the upper areas of the villus surface. Fluorescent bacteria were not seen in the lamina propria or in the crypt area in any part of the small intestine. The results of the immunofluorescence studies for the two 505 organisms and the B44 parent *E. coli* were similar with homologous OK antiserum and K99 antiserum except that there was much more fluorescence with the OK antisera compared with the K99 antiserum.

TABLE III. Viable *E. coli* in Wall Scraping and Contents of the Small Intestine of Calves Given an Oral Dose of 10^{11} Enteropathogenic *E. coli* Strain B44 or the K99⁻ Form of B44

Total *E. coli* ($x10^9$)	Calves given B44			Calves given K99⁻ form		
	1	2	3	1	2	3
Anterior[a]						
Wall	80	33	82	0.006	0.01	0.02
Contents	29	16	29	0.02	0.07	0.07
Middle[a]						
Wall	330	280	350	0.3	0.1	0.01
Contents	138	26	37	0.4	0.04	0.8
Posterior[a]						
Wall	420	400	560	1.8	1.3	1.2
Contents	170	30	40	1.4	2.6	1.3

[a]Anterior, middle and posterior areas of the small intestine

In calves infected with the K99⁻ form of B44, fluorescing organisms were seen in sections from the middle and lower small intestine when homologous OK antiserum was used. In the lower small intestine the bacterial layer was observed to cover only parts of the villi and less than half the villi were colonized whereas in sections from the middle small intestine fewer organisms were seen in some sections and no bacterial adhesion was noted in others.

DISCUSSION

The failure of the acapsular mutant of *E. coli* strain 505 to attain large numbers in the small intestine and cause diarrhea indicate that the capsule of this strain is involved in colonization of the small intestine of calves. The FA staining studies provide evidence that the capsule of this EPEC strain may act as an adhesive substance involved in attachment of the organisms to the intestinal epithelium. In all the calves infected with the parent strain 505 a layer of fluorescing organisms covered the majority of the villi from the tip to the base in the middle and lower small intestine (Fig. 3), whereas no fluorescing organisms were observed in any of the sections examined from the calves infected with the acapsular mutant (Fig. 4). The FA results are in good agreement with the data provided by viable counts which showed that loss of the capsule from strain 505 resulted in a decline in colonization abilities so that it did not reach high numbers and did not colonize any part of the small intestine in any of the calves (Fig. 1, Table II). The FA studies with both parent strains are similar to those reported by Logan and coworkers (11).

The data from the viable counts indicate that the numbers of the acapsular mutant were always approximately three logs lower than those of the parent strain in all areas of the small intestine. Furthermore, 19-43% of the K⁻ organisms were associated with the wall while 69-84% of the K⁺ organisms were associated with the wall. These results are consistent with earlier studies of Nagy *et al* (15) and Smith and Huggins (19) who found that the capsule of some porcine and bovine enteropathogenic *E. coli* strains was an essential virulence factor required for intestinal colonization and expression of full pathogenicity. Their results indicated that removal of the capsule from these *E. coli* strains significantly reduced their proliferative and colonizing abilities.

It is not known whether the reduced viable counts of the K⁻ mutant recovered from all areas of the small intestine compared to the parent strain were due to a loss of adhesive properties of this strain. Previous studies (2,6,7) showed that the capsule of invasive *E. coli* strain enhanced virulence by resistance to phagocytosis and inhibition of the complement dependent bactericidal activity of serum. It is possible that the capsule of the noninvasive bovine enteropathogenic *E. coli* strain 505 plays a similar protective role against the host defense mechanisms operative in the intestine of calves.

In contrast to the comparative consistency of *E. coli* strain B44 in causing diarrhea in all the calves, failure of its K99⁻ form to induce diarrhea in two of the three calves indicates that the K99 antigen was an important factor in colonization of the small intestine. The data from the total viable counts (Fig. 1) demonstrate that the K99⁻ form proliferated to a lesser extent than did the K99⁺ parent strain particularly in the anterior and middle small intestine where counts were two to three logs lower than those of the parent strain, and more organisms were recovered from the intestinal contents than from the wall preparations. However, the K99⁻ form proliferated to moderately large numbers (10^{10}) in the lower small intestine with fewer organisms associated with the wall (44%) than in the K99⁺ form (85%). Fluorescent antibody staining showed that K99⁻ organisms colonized the lower small intestine to a lesser extent than did the K99⁺ parent. These results are in general agreement with previous reports (18,19) on the importance of the K99 antigen in intestinal colonization of calves and lambs. Removal of the K99 antigen from wild calf and lamb enteropathogenic *E. coli* strains significantly reduced their enteropathogenicity and colonizing ability.

In this study, there was considerable evidence to support an important role for the capsular polysaccharide in the colonization of the small intestine of the calf by EPEC. It may be that the K99 pili and the polysaccharide capsule act in concert to anchor the bacteria to the intestinal epithelial cells. Alternatively, or additionally, the capsule may be important for the growth of the bacteria in the small intestine. Recently, Renault and his associates (16) have confirmed that most EPEC isolated from young diarrheic calves have an A type capsular polysaccharide, K99 pili and ST and have suggested that A type capsular polysaccharide should be considered a virulence factor in bovine EPEC implicated in enteric colibacillosis in young calves.

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