INFECTION AND IMMUNITY, Apr. 1976, p. 1214-1220

Vol. 13, No. 4
Printed in U.S.A.

Colonization of Porcine Small Intestine by *Escherichia coli*: Ileal Colonization and Adhesion by Pig Enteropathogens That Lack K88 Antigen and by Some Acapsular Mutants

B. NAGY,[1] H. W. MOON, AND R. E. ISAACSON*

Agricultural Research Service, U.S. Department of Agriculture, National Animal Disease Center, and Department of Veterinary Pathology, College of Veterinary Medicine, Iowa State University, Ames, Iowa 50010*

Received for publication 28 November 1975

Seven K88-negative porcine enteropathogenic *Escherichia coli*, representing three different serogroups, caused severe diarrhea and characteristically colonized the ileum, but not the jejunum, of intragastrically exposed newborn pigs. Bacterial counts of intestinal contents and wall, fluorescence, and scanning electron microscopy all suggested that these strains colonized the ileum by adhesion to the villous epithelium. However, in ligated intestinal loops, these enteropathogenic *E. coli* strains adhered to jejunal epithelium as well as to ileal epithelium. Acapsular (K−) mutants, derived from one of the principal strains, retained their colonizing and adhesive abilities, whereas K− mutants from three other enteropathogenic *E. coli* strains did not. It is suggested that: (i) these K88-negative enteropathogenic *E. coli* colonize the ileum by adhesion, and (ii) the adhesion of some K88-negative strains is mediated by surface factors other than, or in addition to, the polysaccharide K antigen.

In the pathogenesis of enterotoxic diarrheal diseases caused by *Escherichia coli*, two major independent attributes of microbial virulence are involved. These are the abilities to produce enterotoxin and to colonize the small intestine. Colonization of the small intestine requires that enteropathogenic *E. coli* (EEC) multiply in this area and resist the wash-out effects of peristaltic and villous motility. This could be accomplished by either adhesion of the EEC to the intestinal epithelium or, alternatively, suppression of peristaltic and villous motility.

Correlations between the adhesive ability of *Vibrio cholerae* (5), *Streptococcus pyogenes* (3), *Streptococcus mutans* (13), *E. coli* (4, 16), and *Neisseria gonorrhoea* (19) and their ability to colonize the alimentary and genital mucosae have been described. Certain nonpathogenic bacteria of the normal flora in some parts of the alimentary tract of chickens (6) and mice (15) also apparently colonize by adhering to the epithelium.

The plasmid-coded, pilus-like surface antigen K88 is carried by several porcine EEC and confers the ability to colonize porcine small intestine by adhesion to the epithelium (1, 2, 14, 16, 17). This adhesiveness can be demonstrated both in vitro and in vivo (8, 20). There are, however, several EEC strains that do not possess K88 and still they are enteropathogenic for pigs (9, 11, 12). Such strains have been less intensively investigated than the K88-carrying ones, and the data that are available led to the hypothesis that at least some of them colonize by adhesion (2, 7), which is mediated by surface structures other than K88. The experiments reported here utilized several K88-negative porcine EEC strains in tests of this hypothesis.

[1] Visiting scientist from the Veterinary Institute, Szombathely, Hungary.

MATERIALS AND METHODS

Strains of *E. coli*. The EEC strains lacked K88 antigen (Table 1) and were isolated from naturally occurring cases of porcine enteric colibacillosis, whereas the non-enteropathogenic *E. coli* (NEEC) strains used as negative controls were isolated from normal piglets. From four of the EEC strains, seven acapsular (K−), O agglutinable mutant strains were derived (18). Test strains were inoculated in Trypticase soy broth (BBL) and incubated overnight at 37 C without shaking.

Intragastric inoculations. Four to six unfed, hysterectomy-derived, colostrum-deprived, 3- to 10-h-old pigs from at least two different herds were used to test each strain. The pigs were kept in individual isolation units and weighed at the time of inoculation and 16 h later. Prior to inoculation, each was given 20 ml of normal swine serum intraperitoneally and then inoculated intragastrically via stomach tube with 4×10^8 to 5×10^9 bacteria in 20 ml of fresh Trypticase soy broth. Sixteen hours postinocula-

tion, the clinical status of the pigs was recorded. They were then killed and necropsied. A 10-cm segment of jejunum (20 to 30 cm distal to the Ligamentum Trietz) and a 10-cm segment of ileum (15 to 25 cm proximal to the ileocecal valve) were removed from each pig. The content of each segment was washed out with 10 ml of warm (37 C) 0.3% peptone water introduced intraluminally via an 18-gauge needle under pressure from a manually operated 10-ml syringe. The peptone water was referred to as "content," and the washed segment was referred to as the "wall." The number of viable test strain *E. coli* in the contents and on the wall was determined as described earlier (2). Bacterial counts from the content and wall were added to give the total number of viable *E. coli*/10-cm segment. Segments with 10^8 or more total test strain *E. coli* were defined to be colonized. As dilutions made in counting did not permit recovery of *E. coli* if there were less than 10^5/segment, those from which the test strain was not recovered were recorded as having 10^5. For morphological studies, additional segments of ileum and jejunum were taken next to those used for bacterial counts.

Ligated intestinal loops. Ligated intestinal loops were prepared in hysterectomy-derived, colostrum-deprived piglets as described elsewhere (2) and inoculated with 3×10^8 or 10^7 bacteria of the test strains per loop. The pigs were killed and necropsied 3 to 6 h postexposure. Each strain was tested in at least three pigs, representing three different herds. Bacterial counts and morphological studies were done as above.

Association index. The degree of association between intestinal epithelium and the test *E. coli* strain was determined morphologically as described earlier (2), with the modification that only fluorescence microscopy was done. Association indexes were determined only on those segments regarded as colonized ($\geq 10^8$ *E. coli*/10-cm segment) and evaluated from 1 through 5 (as low, <3; intermediate, ≧3 to 4; and high, >4).

Scanning electron microscopy. Samples for electron microscopy were taken from ileal segments of one to two pigs colonized by EEC strains 431, 340, or 74-5208 and fixed in glutaraldehyde (2.5% in 0.1 M cacodylate, pH 7.3). Samples were rinsed in four changes of 0.04 M phosphate-buffered saline (pH 7.2), dried in increasing concentrations of acetone, and coated with 99.9% gold. They were then examined in a Cambridge Stereoscan Mark II A scanning electron microscope at 35° tilt with an accelerating voltage of 20 kV.

Statistical analysis. Analyses of variance were performed on the common logarithms of the bacterial counts and on the weight losses expressed as percentages of the initial body weights. When significant effects were detected, linear comparisons were made to determine the nature of the effects.

RESULTS

Intragastric inoculations. Severe diarrhea and weight loss developed within 16 h postexposure in pigs inoculated with six of the seven EEC strains (Table 2). These EEC-exposed piglets lost an average of 16.4% of their body weight during the 16-h observation period and one of them died. Strain 381 caused diarrhea less frequently and less severe weight loss than the other EEC, although it had comparable cultural and serotypic characteristics to the more virulent EEC strain 74-5208. None of the NEEC strains caused diarrhea, and the weight loss of piglets inoculated with these strains was an average of 5.2% of their initial body weight, significantly ($P > 0.05$) less than that of EEC-inoculated pigs.

TABLE 1. *Parental and mutant Escherichia coli strains used*

Groups	Designation		Serotype
	Parents	K− mutants	
EEC	431		O101:K30(A):NM
		I25	O101:K−:NM
		I26	O101:K−:NM
	613		O101:K30(A):NM
	1351		O101:K30(A):NM
	987		O9:K:(A):NM
		I27	O9:K−:NM
	340[a]		O9:K:(A):NM
		I30	O9:K−:NM
		I31	O9:K−:NM
	74-5208[b]		O20:K+:NM
		I32	O20:K−:NM
		I33	O20:K−:NM
	381		O20:K+:NM
NEEC	123		O43:K−:H28
	124		O8:K50(A):H19
	252		O13:K−:H11

[a] Provided by E. M. Kohler, Ohio.
[b] Provided by R. P. Ellis, South Dakota.

Ileal segments were colonized in 35 of 38 EEC-exposed pigs but only in three of 18 NEEC-exposed pigs. The three noncolonized segments of the EEC-exposed group were from pigs exposed to strain 381. There were 10^5 to 10^{10} EEC/jejunal segment and 10^7 to 10^{11} EEC/ileal segment from EEC-exposed pigs, and there were 10^5 to 10^7 NEEC/jejunal segment and 10^5 to 10^9 NEEC/ileal segment from the NEEC-exposed pigs. The difference between EEC and NEEC was significant in both jejunal ($P > 0.05$) and ileal ($P > 0.01$) counts. The numbers of ileal EEC were significantly ($P > 0.05$) greater than jejunal EEC. However, the differences in the numbers of NEEC between ileum and jejunum were not significant ($P = 0.18$).

The distribution of the test strains between the washed intestinal wall and the content is shown in Table 2 (Fig. 1). The mean percentage of all EEC remaining with the ileal wall after

TABLE 2. *Incidence of diarrhea, weight loss, percentage of the test E. coli strains remaining with the intestinal wall after washing, and association indexes of ileal segments of hysterectomy-derived, colostrum-deprived piglets 16 h post-intragastric exposure to E. coli*

E. coli strains	No. of pigs diarrheal/inoculated	Wt loss (% of initial body wt)	% of total viable *E. coli* remaining with the wall after washing		Association indexes of colonized[a] ileal segments
			Jejunum	Ileum	
1351	6/6	22.28 ± 4.23[b]	63.16 ± 36.09[b]	80.53 ± 8.73[b]	4.47 ± 0.76[b]
613	6/6	21.73 ± 2.63	57.41 ± 30.68	57.97 ± 27.84	4.70 ± 0.27
431	4/4	19.80 ± 4.89	72.32 ± 20.58	69.96 ± 36.21	4.42 ± 0.65
987	4/4	14.57 ± 0.09	48.55 ± 25.55	93.28 ± 4.21	3.92 ± 0.38
340	6/6	18.13 ± 4.31	72.13 ± 26.84	89.54 ± 11.53	4.78 ± 0.29
74-5208	6/6	14.34 ± 5.40	49.15 ± 31.82	85.29 ± 6.53	4.84 ± 0.23
381	4/6	3.72 ± 1.34	72.63 ± 17.54	53.02 ± 21.29	2.70 ± 1.01
123	0/6	3.54 ± 1.63	64.18 ± 22.07	50.35 ± 29.76	1.71 ± ND[c]
124	0/6	2.99	66.63 ± 26.12	49.68 ± 27.44	ND
252	0/6	8.99 ± 7.86	59.11 ± 14.51	39.65 ± 32.94	ND

[a] Segments containing $\geq 10^8$ test strain *E. coli* were recorded as colonized.
[b] Means and standard deviations.
[c] ND, Not determined.

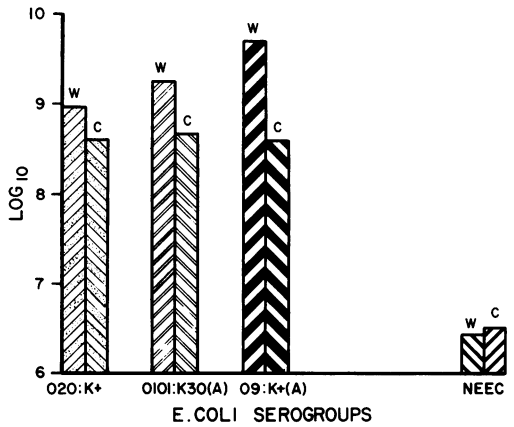

FIG. 1. *Average number of viable E. coli remaining on wall of washed ileal segments (W) and in the content (C) from pigs exposed to EEC strains belonging to serogroups O20:K+, O101:K30(A), and O9:K(A), compared with those from NEEC-exposed pigs.*

washing (75.6%) exceeded that for the NEEC (46.6%). This difference between wall and content was significant ($P = 0.02$) in the ileum but not in the jejunum of EEC-inoculated pigs, and there was no significant difference ($P = 0.30$) between wall and content in the jejunum or ileum of the pigs inoculated with NEEC.

The association indexes were in general agreement with the viable *E. coli* counts, in that the intestinal segments colonized by EEC had high (or occasionally intermediate) association indexes (Table 2). Most cells of the test EEC strains were found closely associated to the epithelial cells, penetrating deeply into the intervillous spaces and covering the villous surfaces from tips to bases. None of the EEC strains were found characteristically in the crypts of Lieberkühn. The washed segments had less *E. coli* in the lumen, but other than that they looked essentially the same as the unwashed ones. The EEC strains in ileal segments were distributed like the K88-possessing EEC previously described (1, 2, 8) (Fig. 2). The NEEC could only be evaluated morphologically when they colonized the ileum. In those three segments they were found mainly in the lumen and occasionally in contact with the epithelial cells, resulting in low association indexes (Table 2).

In most segments examined via scanning electron microscopy, there were microcolonies of 10 to 50 bacterial cells scattered over the villous epithelium. Sometimes most of the villous surface was covered with the EEC (Fig. 3). The bacteria were in close proximity and apparently adhering side-on to the microvilli in most cases. Strains 431, 340, and 74-5208 were all distributed similarly.

The surfaces of the bacteria were granular and rough, particularly those of strain 340. Besides the granular surface, strain 431 also showed mucus-like strands connecting the bacteria with each other and occasionally with microvilli (Fig. 4). We were not able to visualize these strands on the other strains.

Ligated intestinal loop experiments. There were no consistent differences in association indexes of jejunal and ileal loops inoculated with EEC. Strains of the serogroup O101:K30(A) had generally high indexes, whereas the other EEC had intermediate in-

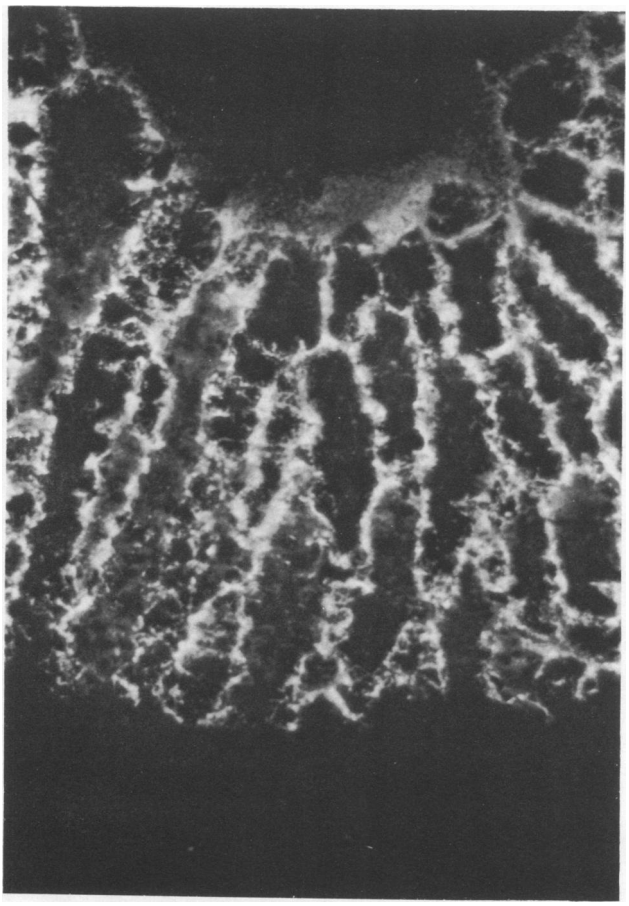

FIG. 2. *E. coli 987 adhering to the epithelium along the entire length of villi (association index, 5.0). Fluorescent antibody-stained, frozen section of ileum of newborn pig 16 h postexposure.*

dexes in both jejunum and ileum. One of the NEEC strains (*E. coli* 124) had intermediate indexes; the other (*E. coli* 252) had low association indexes in both locations.

Intragastric exposure to K− mutants. The colonizing abilities of acapsular (K−) mutants derived from EEC strains 431, 987, 340, and 74-5208 were determined (Table 3). Two groups of K− mutants can be distinguished on the basis of their ability to cause weight loss and colonization. One of them is composed of K− mutants of strain 431, and the other is exposed by those of 987, 340, and 74-5208.

The numbers of *E. coli* per ileal segment and the weight loss in pigs exposed to the two mutants from strain 431 were not significantly different from those of pigs exposed to the parent. Ten of 12 pigs exposed to these two mutants were colonized and had intermediate to high association indexes in ileum (Fig. 5). Whereas all six pigs exposed to the parent strain developed diarrhea, only 10 of 12 pigs exposed to mutants of strain 431 did so.

In contrast to those from strain 431, mutants from the other three strains all caused significantly ($P > 0.01$) less weight loss and attained significantly ($P > 0.01$) lower numbers per ileal segment than did their parent strains. Four of the five mutants from these three strains caused diarrhea less frequently than the parent strains. Mutants I27 and I33 had the greatest decrease in colonizing abilities in comparison with their parents and represented a sharp contrast to the mutants of strain 431.

Distribution of the test strains between washed intestinal wall and content was tested in pigs inoculated with the K− mutants of strains 431 and 987. Washed segments colonized by mutants of strain 431 had about 1-log-higher counts of the test strain remaining with the intestinal wall than in the content, whereas there was no major difference between wall and

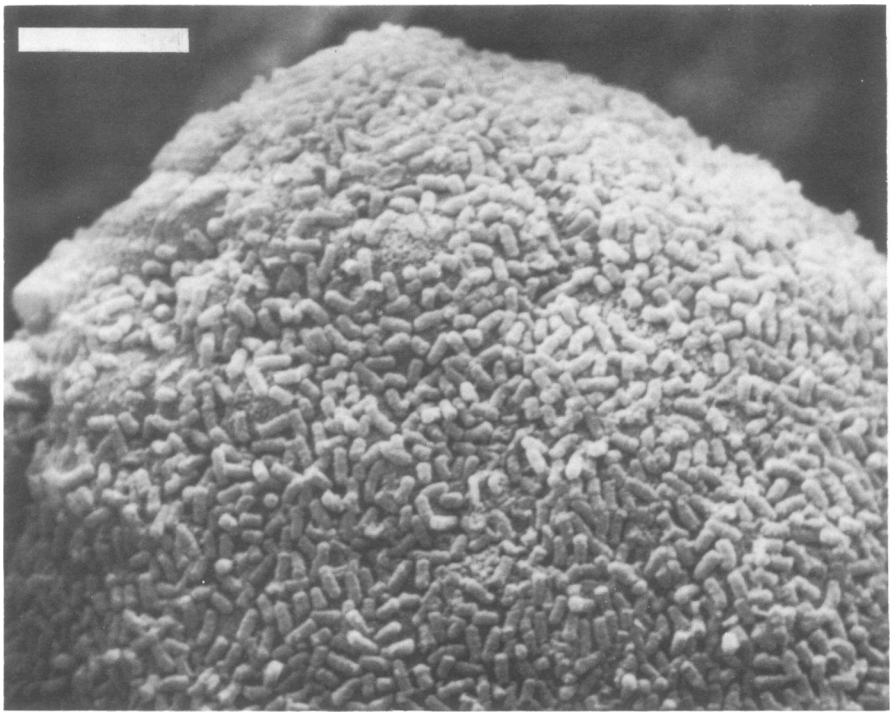

FIG. 3. *Strain 431 EEC adhering to the surface of ileal epithelium at the tip of a villous showing mainly side-on adhesion. Scanning electron micrograph. Bar equals 10 μm.*

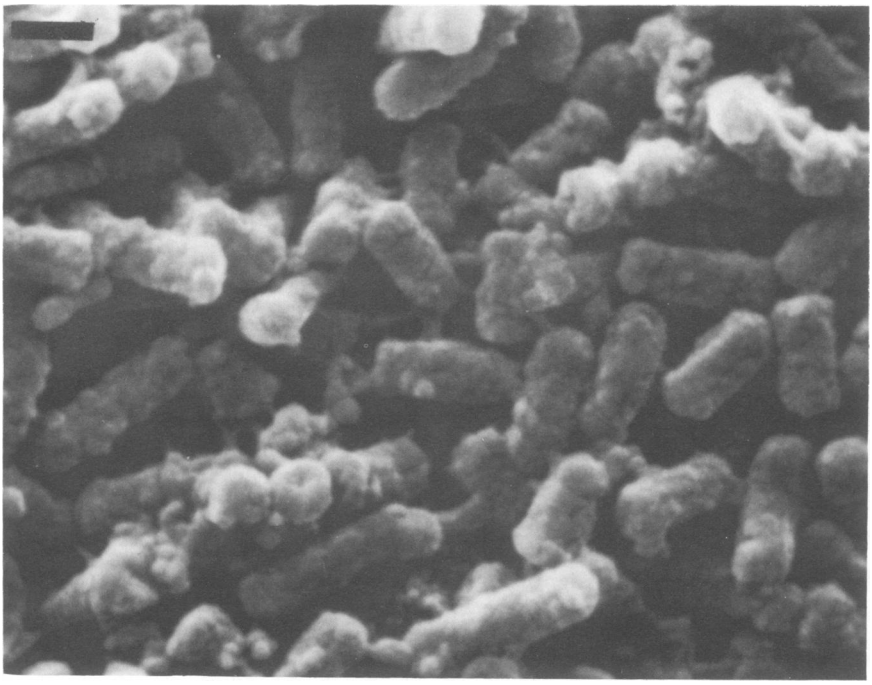

FIG. 4. *Strain 431 EEC cells have granular, rough surfaces and strands extending between some bacteria and towards the microvilli. Scanning electron micrograph. Bar equals 1 μm.*

TABLE 3. *Results of intragastric inoculation of newborn pigs with parental EEC and their K− mutants*[a]

E. coli strains[b]		No. of pigs diarrheal/inoculated	Wt loss (% of initial body wt)	Log_{10} of total viable *E. coli* of 10-cm ileal segments
Parents	Mutants			
431		6/6	16.40 ± 2.23[c]	9.29 ± 0.34[c]
	I25	6/6	13.46 ± 5.68	9.01 ± 0.94
	I26	4/6	15.40 ± 6.20	8.19 ± 2.00
987		6/6	14.40 ± 3.21	9.45 ± 0.23
	I27	3/6	9.26 ± 3.14	6.50 ± 0.75
340		6/6	17.95 ± 3.18	9.76 ± 0.60
	I30	6/6	9.71 ± 2.17	7.85 ± 0.91
	I31	4/6	10.15 ± 2.98	7.65 ± 0.81
74-5208		6/6	17.31 ± 4.03	9.65 ± 0.29
	I32	4/6	10.91 ± 3.29	7.87 ± 1.52
	I33	2/6	8.35 ± 3.72	6.97 ± 1.24

[a] Clinical observations and total viable counts of the test *E. coli* strains in the ileum of hysterectomy-derived, colustrum-deprived piglets 16 h postexposure.

[b] Parent and mutant strains were tested in littermate pigs.

[c] Means and standard deviations.

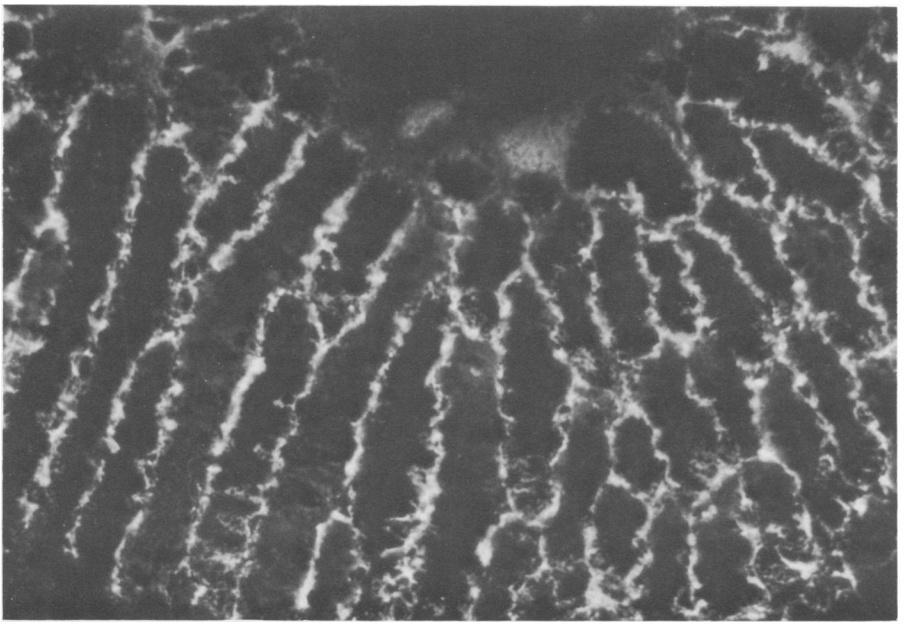

FIG. 5. *E. coli I25, the K− mutant of EEC 431, adhering to the ileal villous epithelium of an intragastrically exposed pig, showing high association index 16 h postexposure. Fluorescent antibody-stained, frozen section.*

content in pigs inoculated with I27, indicating that this mutant had lost its adhesive as well as colonizing abilities.

The K− mutants I25, I26, and I27 were tested in ligated intestinal loops of pigs along with their K+ parents and all were found to be enterotoxigenic.

DISCUSSION

Based on the results of the differential bacterial counts of washed segments and their content, as well as immunofluorescence and scanning electron microscopy, we conclude that these EEC strains, which do not possess the K88 antigen, do colonize the ileum and adhere to ileal epithelium of the newborn pigs. This adhesion is microscopically indistinguishable from that described for K88-containing EEC but differs in its preference for ileum in that K88-possessing EEC colonize both jejunum and ileum (1, 8).

One might conclude that the jejunal epithelium is resistant to adhesion by K88-negative EEC. The results of the ligated loop experi-

ments indicated, however, that this was not the case. In the presence of the same number of bacteria, there were high association indexes in both jejunum and ileum. These observations could be explained if the adhesive forces of K88-carrying strains are stronger than those of K88-negative EEC and if one assumes that peristaltic and villous motilities of jejunum are more vigorous than those of ileum but effectively abolished by creating ligated intestinal loops.

Adhesion by K88-negative strains also differs from that by K88-positive strains in that the latter could be reproduced in vitro, whereas the former could not (8, 20).

Our results confirm and extend those of others (2, 7, 9) in providing evidence for in vivo adhesive characteristics of several serologically distinct K88-negative EEC.

There is evidence that capsular material is important for the survival and reproduction of *E. coli* in the urinary tract (10). Some K− mutants from EEC strains retained their ileal colonizing and adhesive characteristics; others did not (Table 3). As the K− mutants retained their enterotoxigenicity, the decreased colonizing abilities of K− mutants as opposed to parent strains could be interpreted to indicate that the polysaccharide antigen of such strains confers colonizing ability. However, retention of colonizing and adhesive abilities by the mutants from strain 431 demonstrated that polysaccharide K antigen is not an absolute requirement for these attributes. Furthermore, the K88-negative EEC studied here were piliated, and there is evidence that pili contributed to the colonizing abilities of some of these strains (Isaacson et al., submitted for publication). It may be that both polysaccharide K antigens and pili contribute to the adhesive and colonizing abilities of EEC.

ACKNOWLEDGMENTS

We thank Rebecca Jensen and Gary Witmer for excellent technical assistance and Gene Hedberg, Tom L. Glasson, and Wayne Romp for providing illustrative support. We also thank Gordon D. Booth for statistical analyses and Herbert S. Wright (USDA-APHIS) and Frits Ørskov (Statens Seruminstitut, Copenhagen) for the serological designation of some of our strains. Samples for scanning electron microscopy were prepared and photographed by Joe Gallagher, whose contribution is gratefully acknowledged.

This work was supported by ARS-USDA, and U.S. Army Medical Research and Development Command grant no. DADM 17-17-C-5014.

LITERATURE CITED

1. **Arbuckle, J. B. R.** 1970. The location of *Escherichia coli* in the pig intestine. J. Med. Microbiol. 3:333-340.
2. **Bertschinger, H. U., H. W. Moon, and S. C. Whipp.** 1972. Association of *Escherichia coli* with the small intestinal epithelium. I. Comparison of enteropathogenic and nonenteropathogenic porcine strains in pigs. Infect. Immun. 5:595-605.
3. **Ellen, R. P., and R. J. Gibbons.** 1972. M protein-associated adherence of *Streptococcus pyogenes* to epithelial surfaces: prerequisite for virulence. Infect. Immun. 5:826-830.
4. **Evans, D. G., R. P. Silver, D. J. Evans, Jr., D. G. Chase, and S. L. Gorbach.** 1975. Plasmid-controlled colonization factor associated with virulence in *Escherichia coli* enterotoxigenic for humans. Infect. Immun. 12:656-667.
5. **Freter, R.** 1969. Studies on the mechanism of action of intestinal antibody in experimental cholera. Tex. Rep. Biol. Med. 27:299-316.
6. **Fuller, R., and B. E. Brooker.** 1974. Lactobacilli which attach to the crop epithelium of the fowl. Am. J. Clin. Nutr. 27:1305-1312.
7. **Hohmann, A., and M. R. Wilson.** 1975. Adherence of enteropathogenic *Escherichia coli* to intestinal epithelium in vivo. Infect. Immun. 12:866-880.
8. **Jones, G. W., and J. M. Rutter.** 1972. Role of the K88 antigen in the pathogenesis of neonatal diarrhea caused by *Escherichia coli* in piglets. Infect. Immun. 6:918-927.
9. **Kohler, E. M.** 1974. Protection of pigs against neonatal enteric colibacillosis with colostrum and milk from orally vaccinated sows. Am. J. Vet. Res. 35:331-338.
10. **McCabe, W. R., P. C. Carling, S. Bruins, and A. Greely.** 1975. The relation of K-antigen to virulence of *Escherichia coli*. J. Infect. Dis. 131:6-10.
11. **Moon, H. W., D. K. Sorensen, and J. H. Sautter.** 1968. Experimental enteric colibacillosis in piglets. Can. J. Comp. Med. 32:493-497.
12. **Moon, H. W., D. K. Sorensen, J. H. Sautter, and J. M. Higbee.** 1966. Association of *Escherichia coli* with diarrheal disease of the newborn pig. Am. J. Vet. Res. 27:1007-1011.
13. **Mukasa, H., and H. D. Slade.** 1974. Mechanism of the adherence of *Streptococcus mutans* to smooth surfaces. III. Purification and properties of the enzyme complex responsible for adherence. Infect. Immun. 10:1135-1145.
14. **Ørskov, I., and F. Ørskov.** 1966. Episome-carried surface antigen K88 of *Escherichia coli*. I. Transmission of the determinant of the K88 antigen and influence on the transfer of chromosomal markers. J. Bacteriol. 91:69-75.
15. **Savage, D. C., and R. U. H. Blumershine.** 1974. Surface-surface associations in microbial communities populating epithelial habitats in the murine gastrointestinal ecosystem: scanning electron microscopy. Infect. Immun. 10:240-250.
16. **Smith, H. W., and S. Halls.** 1968. The production of oedema disease and diarrhoea in weaned pigs by the oral administration of *Escherichia coli:* factors that influence the course of the experimental disease. J. Med. Microbiol. 1:45-59.
17. **Smith, H. W., and M. A. Linggood.** 1971. Observations on the pathogenic properties of the K88, HLY and ENT plasmids of *Escherichia coli* with particular reference to porcine diarrhoea. J. Med. Microbiol. 4:467-485.
18. **Smith, T., and G. Bryant.** 1927. Studies on pathogenic *E. coli* from bovine sources. II. Mutations and their immunological significance. J. Exp. Med. 46:133-140.
19. **Swanson, J. S., J. Kraus, and E. C. Gotschlich.** 1971. Studies on gonococcus infection. I. Pili and zones of adhesion: their relation to gonococcal growth patterns. J. Exp. Med. 134:886-906.
20. **Wilson, M. R., and A. W. Hohmann.** 1974. Immunity to *Escherichia coli* in pigs: adhesion of enteropathogenic *Escherichia coli* to isolated intestinal epithelial cells. Infect. Immun. 10:776-782.