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In Vivo Emergence of Enterotoxigenic *Escherichia coli* Variants Lacking Genes for K99 Fimbriae and Heat-Stable Enterotoxin

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Neonatal pigs were inoculated with porcine enterotoxigenic *Escherichia coli* 431, which carries genes for K99 fimbriae and STaP enterotoxin. Colonies of strain 431 were recovered from feces of pigs for up to 17 days after inoculation and tested for hybridization with gene probes for K99 and STaP. Variants of strain 431 that did not hybridize with the probes were considered to have lost the genes. Variants were recovered from 10 of 13 suckling pigs that survived the infection. Only 0.4% of the isolates recovered during the first 2 days after inoculation were variants. Of the isolates recovered 3 to 5 days after inoculation, 20 to 36% were variants. Variant colonies were detected more frequently among pigs in some litters than in others. The litter with the highest number of variant-shedding pigs had the dam with the highest titer of K99 antibody in her colostrum. Variants also occurred in colostrum-deprived, artificially reared pigs. However, the number of variants detected was lower and they occurred later in the course of the infection in colostrum-deprived pigs than in suckling pigs. More variants were detected and they were detected earlier in colostrum-deprived pigs fed anti-K99 monoclonal antibody than in controls fed anti-K88 monoclonal antibody. Loss of STaP appeared to be secondary to loss of K99 in that some variants lacked only K99 ($K99^-$ $STaP^+$) and some lacked both genes ($K99^-$ $STaP^-$), but none was of the $K99^+$ $STaP^-$ type. Our results confirmed reports of gene loss from enterotoxigenic *E. coli* during infection. They are consistent with the hypothesis that variants emerge under in vivo selection pressure of K99 antibody and with the speculation that gene loss may be an important component of protection in vaccinated populations. However, the emergence of variants did not appear to play a major role in the recovery of individual pigs from clinical disease.

Fimbriae (pili) and enterotoxins are virulence factors of enterotoxigenic *Escherichia coli* (ETEC) (1, 15). Fimbriae mediate adherence of *E. coli* to host intestinal epithelium, and enterotoxins stimulate intestinal secretion of water and electrolytes, resulting in diarrhea. Some ETEC strains of the K88 (F4) and K99 (F5) fimbrial antigen types lose these antigens (and, apparently, the plasmids carrying the genes encoding them) during experimental infections in mice (5). Loss of K88 antigen also occurs during infections in pigs, which are natural hosts for $K88^+$ ETEC (17, 18). A human ETEC strain (6) and an enteropathogenic *E. coli* strain (7) have been shown to lose genes encoding heat-labile enterotoxin and an adherence factor, respectively, during infection in humans.

Loss of K88 in pigs is thought to be caused by antibody, because it was detected in immunized pigs but not in control pigs (18) and because it can be induced in vitro by growth in immune colostrum (8, 17, 18). It has been suggested that the antibodies involved in loss of K88 are not K88 specific but rather are directed against a novel antigen(s) and act by a novel mechanism which also impedes the spread of plasmids encoding drug resistance (8, 17, 18; M. Lingood and P. Porter, Proc. Int. Pig Vet. Soc. 19th Congr., p. 146, 1986). However, loss of K88 and K99 occurs during growth in specific antibody in vitro (13, 17; C. Paniagua, P. Rubio, M. Alvarez, and S. Suárez, Proc. Int. Pig Vet. Soc. 19th Congr., p. 132, 1986), and K88 antibody selects for cells that spontaneously lose the K88 plasmid during growth in vitro (13). We were interested in the phenomenon of in vivo gene loss by ETEC because of the implication that some of the protection in vaccinated populations might be due to a "disarming" of the pathogens before they are excreted into the environment. The emergence of such disarmed bacteria in diseased individuals might also allow the individuals to recover from disease; that is, theoretically, recovery could occur because the infecting bacteria no longer possess the attributes required to maintain the disease.

The objectives of the present study were to determine whether a $K99^+$ porcine ETEC strain would lose genes for K99 and enterotoxin during infection in (i) conventional suckling newborn pigs, (ii) colostrum-deprived pigs, and (iii) colostrum-deprived pigs fed monoclonal antibody (MAb) against the K99 or the K88 antigen. We found that variants lacking the genes emerged in all groups. However, they were detected most frequently and earliest in pigs that received K99 antibody.

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MATERIALS AND METHODS

Bacteria. *E. coli* 431 (O101:K30,99:F41:NM) was used. Strain 431 produces a heat-stable enterotoxin that is active in infant mice (STa) and hybridizes with the STaP gene probe derived from a porcine ETEC strain (11). Before this study, strain 431 was maintained for 21 years on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) in sealed tubes, stored at room temperature in the dark, with a nonselective transfer (without cloning or selection) to fresh medium every 3 years. All inocula came from samples of the same culture, which was prepared and stored at −70°C, as reported previously (12, 19), for more than 1 year. Samples were thawed and diluted in Trypticase soy broth (BBL) just before inoculation into pigs. At least 99.5% of the viable

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bacteria in the inocula contained the genes encoding both K99 and STaP (see below).

Pigs and experimental design. The experiment in suckling pigs involved the newborn pigs of five gilts (primiparous swine). In a separate study (19), four of these gilts received a vaccine containing F41 fimbriae, and the other received a placebo. None of them was vaccinated against K99. Titers of K99 and F41 antibody in colostral whey were determined by enzyme-linked immunosorbent assay of samples collected from the gilts at farrowing (19). When the pigs were 1 to 7 h old, each was inoculated intragastrically with 10^{10} CFU of strain 431 (19). Rectal swabs were collected from the pigs daily from 1 to 5 days postinoculation (p.i.) and examined for variants (see below).

Colostrum-deprived pigs were derived by hysterectomy, housed in individual isolation cages, and fed milk replacer (SPF-Lac; Borden Inc., Hampshire, Ill.). Six colostrum-deprived pigs were used in the first such experiment. Four were inoculated with strain 431 (10^8 CFU per pig, intragastrically) at 2 days of age, one was inoculated at 6 days of age, and the sixth remained noninoculated. Rectal swabs were collected from the pigs daily for 5 days p.i. and then every second day until day 15 p.i.

Eight colostrum-deprived pigs were used in the second experiment. Four pigs received K99 MAb, and four received K88 MAb (10 mg of gamma globulin per pig per day). The MAb was mixed with milk replacer and fed three times per day from 2 through 7 days of age. Each pig was inoculated (10^4 CFU, intragastrically) with strain 431 just before its second MAb feeding at 2 days of age. Rectal swabs were collected daily for 5 days p.i. and every third day until day 17 p.i.

Isolation and identification of variants. Rectal swabs were frozen at −70°C in screw-cap vials for ≤2 months. The swabs were thawed, material from them was suspended in 0.3% peptone water, samples were inoculated onto MacConkey agar plates, and the plates were incubated for 18 to 24 h at 37°C. Colonies that were morphologically similar to those of strain 431 were tested for agglutination in antisera raised against live cells of *E. coli* 431. Up to 10 (depending on availability) colonies of agglutinable bacteria were isolated from each swab, transferred onto Luria-Bertani agar plates (10), and grown for 18 to 24 h at 37°C. The colonies from Luria-Bertani agar (along with control colonies of strain 431 and a negative control strain of *E. coli*) were then transferred onto Whatman 541 paper filters (VWR Scientific, San Francisco, Calif.) and treated to release and denature the DNA as reported previously (9). Gene probes for K99 and STaP DNA were derived and used in DNA colony hybridization tests as reported previously (9). Hybridization tests were repeated on any colony (isolate) that did not hybridize with one or both of the gene probes on the initial test. Isolates that consistently failed to hybridize with one or both of the probes in repeated tests (two or more) were tested further to determine whether they were variants of *E. coli* 431.

The tests used to identify $K99^-$ or $STaP^-$ isolates as variants of strain 431 were carbohydrate fermentation, antibiotic resistance, and serogrouping. Fermentation of adonitol, salicin, and sucrose was tested by growing the isolates at 37°C on nutrient agar containing bromthymol blue as a pH indicator and 1% of the test sugar. In these conditions, strain 431 fermented adonitol after 24 h and salicin after 72 h but did not ferment sucrose. Antibiotic resistance was tested by growth at 37°C for 24 h on nutrient agar supplemented with either 25 μg of streptomycin or 15 μg of tetracycline per ml. Strain 431 grows in these conditions. Isolates were tested for the O101 and K30 antigens by agglutination tests with specific antisera. Isolates that had the same carbohydrate fermentation pattern, antibiotic resistance, and O:K group as strain 431 in the above tests but failed to hybridize with either the K99 or STaP probes were considered to be variants of strain 431 that lacked these genes.

TABLE 1. Titers of K99 and F41 antibodies in colostrum from gilts and response of their suckling pigs for 5 days after oral inoculation with *E. coli* 431 (O101:K30,99:F41:NM)

Gilt no.	Colostral antibody titer		No. of pigs affected/ no. inoculated		
			Diarrhea		Death
	K99	F41	Day 1 p.i.	Day 5 p.i.	
491	0	100	4/4	0/1	3/4
497	0	100	7/8	0/2	6/8
520	400	0	6/8	0/8	0/8
528	0	200	4/4	0/1	3/4
597	100	10	7/7	2/2	5/7

Several samples of the frozen inoculum of strain 431 were also grown directly on MacConkey agar; 200 colonies were transferred to and grown on Luria-Bertani agar, transferred to filter paper, and hybridized with the gene probes. Each of these 200 colonies hybridized with both of the gene probes ($K99^+$ $STaP^+$), indicating that most of the viable bacteria in the inocula contained both genes.

MAb. The K99 MAb was prepared from hybridoma K992 BD4E4 as reported previously (20, 21). The K88 MAb was prepared from hybridoma 21BA13G4. Mice were immunized with K88ab and K88ad antigens extracted by heating bacteria at 65°C for 30 min (23). Hybridoma 21BA13G4 was derived by fusion (21) and found to produce antibody reactive against purified K88ac antigen provided by Isaacson (4). Both MAb were of the immunoglobulin G1 isotype and used as mouse ascites fluid. The dosage of MAb fed was based on the gamma globulin content of the ascites fluid (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis).

Tests for K99 MAb and K99 antigen available (not combined with or blocked by competing MAb or antigen in the system) in feces were conducted on material from rectal swabs. The swabs were thawed, and the feces they contained were suspended (10% [wt/vol]) in buffer (8.0 g of NaCl, 0.2 g of KCl, 1.5 g of Na_2HPO_4 [anhydrous], 0.2 g of KH_2PO_4 [anhydrous], 1.0 ml of Tween 20, water added to make a total of 1,000 ml; pH 7.3 to 7.5). The quantity of K99 MAb in feces of pigs was determined by an enzyme-linked immunosorbent assay with K99 antigen (3) (1 μg/ml in 60 mM carbonate buffer) in the solid phase and peroxidase-conjugated anti-mouse immunoglobulin as the indicator system (25). Results were expressed as micrograms of immunoglobulin per gram of feces, with mouse ascites fluid containing K99 MAb as the standard. The quantity of K99 antigen available in feces was determined by an enzyme-linked immunosorbent assay with the K99 MAb to capture the antigen and peroxidase-conjugated K99 MAb as the indicator system. This assay was standardized against isolated K99 antigen (purity, >95%) (4).

RESULTS

Suckling pigs. The response of suckling pigs to inoculation with *E. coli* 431 is summarized in Table 1. All but 3 of the 31 inoculated pigs developed diarrhea, and 17 of them died of

TABLE 2. Isolation[a] of K99 or STaP variants of ETEC strain 431

Litter no.	Day p.i.	No. of variants/total[b]	No. of pigs with variants/total[c]
491	1	0/4	0/1
	2	0/10	0/1
	3	0/10	0/1
	4	0/10	0/1
	5	0/10	0/1
497	1	0/9	0/1
	2	0/9	0/1
	3	0/9	0/1
	4	0/9	0/1
	5	0/9	0/1
520	1	1/75	1/8
	2	0/64	0/8
	3	15/46	3/8
	4	25/53	6/8
	5	26/55	6/7[d]
528	1	0/8	0/1
	2	0/10	0/1
	3	3/9	1/1
	4	2/9	1/1
	5	2/9	1/1
597	1	0/18	0/2
	2	0/19	0/2
	3	0/17	0/2
	4	0/19	0/2
	5	8/18	1/2

[a] Isolated from rectal swabs from suckling pigs inoculated with *E. coli* 431.
[b] Number of variant colonies/number of colonies tested by DNA-DNA hybridization with gene probes for K99 and STaP.
[c] Number of pigs from which variants were recovered/number of pigs shedding strain 431.
[d] One of the eight pigs in this litter had ceased shedding strain 431 by day 5 p.i.

diarrheal disease by day 5 p.i. Gilt 520 had the highest titer of colostral K99 antibody, and all eight of the pigs in that litter survived.

The results of examination of rectal swabs from 13 of the 14 surviving pigs are summarized in Table 2. Bacteria from a total of 518 selected colonies were agglutinable in antiserum to strain 431; 436 (84%) of the colonies hybridized with both probes ($K99^+$ $STaP^+$), 27 (5%) hybridized with STaP only ($K99^-$ $STaP^+$), and 55 (11%) of them were $K99^-$ $STaP^-$. Several pigs excreted both types ($K99^-$ $STaP^+$ and $K99^-$ $STaP^-$) of variants. None of the colonies hybridized with K99 but not STaP ($K99^+$ $STaP^-$). Thus, a total of 82 presumptive variants of strain 431 were isolated (Table 2). All 82 of the presumptive variants had the same phenotype (carbohydrate fermentation, antibiotic resistance, O101:K30 serogroup) as strain 431.

Variants were recovered from 10 of the 13 pigs examined (Table 2). The percentage of variant colonies isolated varied with time and litter and among pigs within litters. The number of pigs shedding variants increased with time. During the first 2 days p.i., only 1 of 226 (0.4%) colonies tested was a variant (Table 2). The percentage of variants increased to 20% by day 3 and to 36% by day 5 p.i. Variants were not recovered at any time from either of the pigs in litters 491 and 497, nor were they recovered from one of the two pigs in litter 597. In contrast, 23% of the colonies from pigs in litter 520 were variants (Table 2), and variants were recovered from each of the eight pigs in litter 520. Eighty-eight percent of the colonies recovered from pig 4 in litter 520 during days 3 to 5 p.i. were variants. However, only 7% of the colonies recovered from a littermate (pig 8, litter 520) during the same period were variants. The litter (520) that produced the highest percentage of variant colonies (Table 2) was also the litter suckling the gilt with the highest colostral K99 antibody titer and the greatest percentage of surviving pigs (Table 1). The data were skewed toward isolates from pigs suckling gilts with detectable levels of K99 antibody, because most of the surviving pigs were in those two litters. However, gilt 528 did not have detectable K99 antibody, and variants were isolated from the sole surviving pig in litter 528 (Table 2).

Colostrum-deprived pigs. The purposes of the first experiment in colostrum-deprived pigs were to determine whether variants would occur in pigs devoid of antibody and whether any such pigs would survive inoculation with strain 431. Four pigs were inoculated with strain 431 at 2 days of age, and two pigs were left as controls. By day 2 p.i., three of the inoculated pigs had died with diarrhea, and the fourth was recovering. Controls remained healthy. At 6 days of age, one of the controls was also inoculated with strain 431; it subsequently developed diarrhea and survived. The two surviving inoculated pigs and the remaining control pig were tested for variants from days 1 to 15 p.i. (Table 3). Of the 83 colonies of strain 431 recovered from the pig inoculated at 2 days of age, 5 were variants. Variants were first recovered on day 13 p.i. None of 87 colonies of strain 431 recovered from the pig inoculated at 6 days of age was a variant.

The purpose of the second experiment was to determine whether variants would be detectable earlier or in greater numbers in survivors fed K99 MAb than in those fed K88 MAb. Because of the high mortality and late detection (day 13 p.i.) of variants among pigs in the first experiment, in the second experiment pigs were inoculated with a lower dose of strain 431, and observations were extended to 17 days p.i. All of the inoculated pigs developed diarrhea, and one pig in each group died with diarrhea (Table 3). Diarrhea started 1 to 2 days later and terminated 1 to 2 days earlier (Table 3) and death occurred 1 day later (Fig. 1) in the pigs fed K99 MAb than in those fed K88 MAb. Variants of strain 431 were recovered from only one of the three surviving pigs in each group. However, variants were first recovered from K99 MAb-fed pigs at day 3 p.i., and 3.3% of all strain 431 colonies recovered from that group were variants (Table 3). In contrast, only 1 of the 270 (0.4%) colonies of strain 431 recovered from the three K88 MAb-fed pigs was a variant, and it was isolated on day 17 p.i.

All 15 of the presumptive variants isolated during the two experiments had the same phenotype as strain 431. Three of them (one from each variant-producing pig shown in Table 3) hybridized with the STaP probe but not the K99 probe ($K99^-$ $STaP^+$); the other 12 did not hybridize with either probe ($K99^-$ $STaP^-$).

None of the pigs that were fed K99 MAb had detectable levels of K99 MAb in its feces on the first day after inoculation with strain 431 (Table 4). However, two of them had K99 MAb in feces by day 2 p.i., and all three of the pigs that survived had detectable K99 MAb in feces from days 3 to 5 p.i. None of the samples collected (days 1 to 3 p.i.) from the K99 MAb-fed pig that died contained detectable levels of K99 MAb. Samples collected from principals after MAb feeding had been discontinued (days 8 to 17) did not contain detectable levels of K99 MAb (Table 4). None of the samples from controls fed K88 MAb contained detectable K99 MAb (data not shown).

TABLE 3. Effect of K99 MAb on the isolation of K99$^-$ variants of *E. coli* 431 from colostrum-deprived pigs

Inoculation[a]		Antibody[b]	No. of pigs affected/no. inoculated			No. of pigs with variants/total[c]	No. of variants/total[d]	Day (p.i.) of isolation
			Diarrhea		Death			
Age of pigs (days)	CFU		Day 1 p.i.	Day 6 p.i.				
2	10^8	None	4/4	0/1	3/4	1/1	5/83	13
2	0	None	0/1	0/1	0/1	0/1		
6	10^8	None	1/1	0/1	0/1	0/1	0/87	
2	10^4	K99	2/4	0/3	1/4	1/3	9/270	3
2	10^4	K88	4/4	3/3	1/4	1/3	1/270	17

[a] Pigs were inoculated intragastrically with *E. coli* 431 (K99$^+$).
[b] Controls were not fed antibody or were fed K88 MAb. Principals were fed K99 MAb.
[c] See footnote *c* of Table 2.
[d] See footnote *b* of Table 2.

The mean concentrations of available K99 antigen in feces of pigs fed K99 MAb (principals) and K88 MAb (controls) are compared in Fig. 1. The controls maintained comparatively high levels through day 5 p.i., although they tended to drop by day 3 after the death of a pig with one of the highest antigen concentrations. Antigen concentrations of all four principals were lower than those of any of the controls on day 1 p.i., and they remained comparatively low through day 5 among the three principals that survived. However, the mean antigen concentration of principals approached that of controls on days 2 and 3, before dropping to comparatively low levels on day 4 (Fig. 1). This drop followed the death of the principal with the highest antigen concentrations. Antigen concentrations among principals rose to control levels after antibody feeding was discontinued.

DISCUSSION

Our results confirmed reports that ETEC variants lacking genes for virulence emerge during infection (5, 6, 17, 18). They extended such evidence to include a gene for STa enterotoxin and to the gene for K99 in a natural host (the pig). They further demonstrated that some variants lacking the genes encoding K99 and STaP emerged in nonvaccinated, colostrum-deprived pigs. Theoretically, in vivo gene loss by ETEC could occur as the result of some undefined mutagenic effects, or variants could emerge at normal levels of mutation under selective pressures directed against the parental type. There is good evidence that the latter mechanism applies to loss of K88 and K99 in vitro (13).

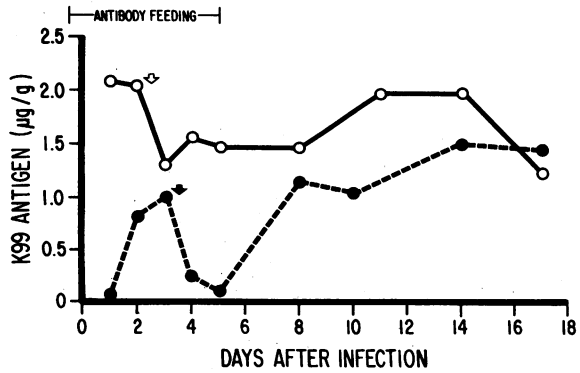

FIG. 1. Mean concentrations of K99 antigen available in feces of pigs inoculated with a K99$^+$ ETEC strain. All pigs were fed MAb for 5 days. Principals (●) were fed K99 MAb, and controls (○) were fed K88 MAb. There were four pigs per group; one pig in each group died at the time indicated by the arrow.

We think the strain 431 variants emerged, at least in part, because of the selection pressure of K99 antibody. Some observations consistent with that hypothesis are as follows: (i) others have shown that K88 antibody selects for K88$^-$ variants in vitro (13; Paniagua et al., Proc. Int. Pig Vet. Soc. 19th Congr.), that K99$^-$ variants of some *E. coli* strains emerge during in vitro growth in K99 antibody (17, 18), and that phenotypic suppression of K88 or K99 is an alternative mechanism for accommodating the in vitro selection pressure of specific antibody (13); (ii) variant colonies were detected most frequently in pigs suckling the dam with the highest titer of colostral K99 antibody (Tables 1 and 2); (iii) variant colonies were detected more frequently and earlier in colostrum-deprived pigs fed K99 MAb than in control pigs (Table 3); and (iv) none of the variants was K99$^+$ STaP$^-$.

However, variants occured in one of three pigs suckling dams that did not have detectable levels of colostral K99 antibody (litter 528, Tables 1 and 2) and in two colostrum-deprived pigs that were not fed K99 MAb (Table 3). It is possible that these variants also occurred as the result of K99 antibody. Colostrum samples recorded as negative (Table 1) may have contained K99 antibody at levels not detectable by the assay used. Variants were not detected until 13 days p.i. in colostrum-deprived pigs that were not fed K99 in MAb. By that time, the pigs had recovered from clinical disease, had presumably developed active local immunity to strain 431, and were probably secreting K99 antibody into their intestines (14). Such porcine K99 antibody would not have been detected by the anti-mouse immunoglobulin reagent used to test for K99 MAb in feces (Table 4).

Variants were detected less frequently in K99 MAb-fed

TABLE 4. K99 MAb in feces of pigs fed K99 MAb and inoculated with K99$^+$ ETEC[a]

Day(s) p.i.	No. of pigs positive/total	MAb (μg of immunoglobulin/g)	
		Mean	Range
1	0/4	0	0
2	2/4	2.16	0–6.50
3	3/4	3.89	0–8.57
4	3/3[b]	4.90	3.83–6.77
5	3/3	5.25	1.66–10.2
8 to 17	0/3	0	0

[a] Pigs were fed MAb from days 0 to 5 p.i.
[b] One pig died, so there were no further samples.

pigs than in pigs suckling dams with colostral K99 antibody. Some possible explanations for this difference are as follows. (i) Antigens other than K99 or factors other than antibody may have been involved. (ii) The polyclonal swine antibody may have been directed against a K99 epitope(s) not recognized by the K99 MAb or may have acted via a mechanism more suited to swine immunoglobulin than to mouse immunoglobulin or to polyclonal antibody than to MAb. (iii) The levels of intestinal K99 antibody attained in the MAb feeding experiment may have been lower than that in suckling pigs, due to the lack of protease inhibitors normally found in colostrum (24). Direct comparisons of K99 antibody levels of suckling and MAb-fed pigs were not made. The K99 MAb-fed pigs did attain detectable levels of K99 MAb in feces in three of the four pigs (Table 4). There was evidence that the K99 MAb did exert some effects, even though one pig died in both the K99 MAb- and K88 MAb-fed groups. During the period of MAb feeding, the levels of available K99 antigen in feces were lower, the onset of diarrhea was later, and the duration of diarrhea was shorter in K99 MAb-fed pigs than in those fed K88 MAb.

Although we interpret our data to suggest that the variants emerged in response to K99 antibody, the data do not exclude the possibility that factors other than antibody or antibody directed against antigens other than K99 contributed to the emergence of the variants. Theoretically, antibody could have contributed to the emergence of variants by suppressing the growth of parental-type bacteria or removing them from the system. It could also have acted by a trivial mechanism, such as agglutination of large numbers of viable parental-type bacteria into clumps (which may have remained in the system and been recognized as single colonies), facilitating detection of variant colonies formed from single bacteria. Our data do not distinguish among these theoretical, non-mutually exclusive mechanisms.

The genes for colonization factor antigen II fimbriae as well as those for heat-labile and STa enterotoxins are located on the same plasmid in some human ETEC strains (16). Loss of genes for colonization factor antigen II and enterotoxins from such strains occurred during storage in vitro, both by loss of the plasmid and by deletions from the plasmid (16). The K99 and STaP genes both occur on the same plasmid in some strains (2). It has recently been shown that the K99 and STaP genes are both on the same plasmid in strain 431 (J. Mainil, F. Bex, P. Dreze, A. Kaeckenbeeck, M. Couturier, Proc. Eur. Congr. Clin. Microbiol., abstr. no. 48, The Hague, The Netherlands, 1987). None of the variants was $K99^+$ $STaP^-$, suggesting that loss of STaP was secondary to loss of K99. This could be explained by selection against K99, with both genes on the same plasmid. Variants that occurred as the result of loss of the plasmid from some isolates would be $K99^-$ $STaP^-$; those that occurred as the result of loss of the K99 gene from the plasmid of other isolates would be $K99^-$ $STaP^+$. Alternatively, STa has been shown to occur as a transposon (22); thus, it is conceivable that STaP is on the K99 plasmid in some progeny and elsewhere in other progeny of strain 431.

It has been suggested that antibody-mediated emergence of variant ETEC strains that lack genes for virulence is an important component of protection in vaccinated populations (1, 8, 17, 18). Our data demonstrate that such variants emerge in nonvaccinated animals. Neither the level of the loss required for a significant protective effect in an animal population nor the level of loss attainable in vaccinated individuals is known. Thus, the importance of gene loss in vaccine development remains speculative.

Variants were detected infrequently during the early stages of the disease (days 1 and 2) and more frequently with time and recovery of the pigs from the clinical disease (Tables 2 and 3). This was consistent with the notion that loss of virulence contributed to recovery from the disease. On the other hand, variants were never detected in some pigs that recovered, some of the pigs that were not excreting detectable levels of variants recovered as rapidly as those that were, and the parental type persisted at detectable levels in all pigs during recovery (Tables 1 to 3). These observations suggest that the emergence of less virulent bacteria (variants) was not a major factor allowing recovery of the pigs. It seems reasonable to speculate that suppression of the $K99^+$ parental type by the antibody led to reduced colonization of the small intestine and, thus, secondarily allowed both recovery of the pigs and emergence of the variants.

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LITERATURE CITED

1. **Gaastra, W., and F. K. de Graaf.** 1982. Host-specific fimbrial adhesins of noninvasive enterotoxigenic *Escherichia coli* strains. Microbiol. Rev. **46**:129–161.
2. **Harnett, N. M., and C. L. Gyles.** 1985. Enterotoxin plasmids in bovine and porcine enterotoxigenic *Escherichia coli* of O groups 9, 20, 64, and 101. Can. J. Comp. Med. **49**:79–87.
3. **Isaacson, R. E.** 1977. K99 surface antigen of *Escherichia coli*: purification and partial characterization. Infect. Immun. **15**: 272–279.
4. **Isaacson, R. E.** 1980. Pili of enterotoxigenic *Escherichia coli*, p. 213–231. *In* S. D. Acres, A. J. Forman, and H. Fast (ed.), Proceedings of the Third International Symposium on Neonatal Diarrhea. Veterinary Infectious Disease Organization, Saskatoon, Saskatchewan, Canada.
5. **Kétyi, I., B. Kuch, and A. Verténvi.** 1978. Stability of *Escherichia coli* adhesive factors K88 and K99 in mice. Acta Microbiol. Acad. Sci. Hung. **25**:77–86.
6. **Lanata, C. F., J. B. Kaper, M. M. Baldini, R. E. Black, and M. M. Levine.** 1985. Sensitivity and specificity of DNA probes with the stool blot technique for detection of *Escherichia coli* enterotoxins. J. Infect. Dis. **152**:1087–1090.
7. **Levine, M. M., J. P. Nataro, H. Karch, M. M. Baldini, J. B. Kaper, R. E. Black, M. L. Clements, and A. D. O'Brien.** 1985. The diarrheal response of humans to some classic serotypes of enteropathogenic *Escherichia coli* is dependent on a plasmid encoding an enteroadhesiveness factor. J. Infect. Dis. **152**: 550–559.
8. **Linggood, M. A., M. L. Ellis, and P. Porter.** 1979. An examination of the O and K specificity involved in the antibody-induced loss of the K88 plasmid from porcine enteropathogenic strains of *Escherichia coli*. Immunology **38**:123–127.
9. **Mainil, J. G., S. L. Moseley, R. A. Schneider, K. Sutch, T. A. Casey, and H. W. Moon.** 1986. Hybridization of bovine *Escherichia coli* isolates with gene probes for four enterotoxins (STaP, STaH, STb, LT) and one adhesion factor (K99). Am. J. Vet. Res. **47**:1145–1148.
10. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
11. **Moon, H. W., R. A. Schneider, and S. L. Moseley.** 1986. Comparative prevalence of four enterotoxin genes among *Escherichia coli* isolated from swine. Am. J. Vet. Res. **47**:210–212.

12. **Morgan, R. L., R. E. Isaacson, H. W. Moon, C. C. Brinton, and C.-C. To.** 1978. Immunization of suckling pigs against enterotoxigenic *Escherichia coli*-induced diarrheal disease by vaccinating dams with purified 987 or K99 pili: protection correlates with pilus homology of vaccine and challenge. Infect. Immun. **22:**771–777.
13. **Nagy, L. K., T. MacKenzie, D. J. Pickard, and G. Dougan.** 1986. Effects of immune colostrum on the expression of a K88 plasmid encoded determinant: role of plasmid stability and influence of phenotypic expression of K88 fimbriae. J. Gen. Microbiol. **132:**2497–2503.
14. **Olsson, E., C. J. Smyth, O. Söderlind, A.-M. Svennerholm, and R. Möllby.** 1986. Development of intestinal antibodies against *Escherichia coli* antigens in piglets with experimental neonatal *E. coli* diarrhoea. Vet. Microbiol. **12:**119–133.
15. **Ørskov, I., and F. Ørskov.** 1983. Serology of *Escherichia coli* fimbriae. Prog. Allergy **33:**80–105.
16. **Peñaranda, M. E., D. G. Evans, B. E. Murray, and D. J. Evans, Jr.** 1983. ST:LT:CFA/II plasmids in enterotoxigenic *Escherichia coli* belonging to serogroups O6, O8, O80, O85, and O139. J. Bacteriol. **154:**980–983.
17. **Porter, P., and M. A. Linggood.** 1983. Novel mucosal antimicrobial functions interfering with the plasmid-mediated virulence determinants of adherence and drug resistance. Ann. N.Y. Acad. Sci. **409:**564–579.
18. **Porter, P., and M. A. Linggood.** 1983. Development of oral vaccines for preventing diarrhoea caused by enteropathogenic *Escherichia coli*. J. Infect. **6:**111–121.
19. **Runnels, P. L., S. L. Moseley, and H. W. Moon.** 1987. F41 pili as protective antigens of enterotoxigenic *Escherichia coli* that produce F41, K99, or both pilus antigens. Infect. Immun. **55:** 555–558.
20. **Sadowski, P. L., S. D. Acres, and D. M. Sherman.** 1983. Monoclonal antibody for the protection of neonatal pigs and calves from toxic diarrhea, p. 93–99. *In* A. Hollaender, A. I. Laskin, and P. Rogers (ed.), Basic biology of new developments in biotechnology. Basic life sciences, vol. 25. Plenum Publishing Corp., New York.
21. **Sherman, D. M., S. D. Acres. P. L. Sadowski, J. A. Springer, B. Bray, T. J. G. Raybould, and C. C. Muscoplat.** 1983. Protection of calves against fatal enteric colibacillosis by orally administered *Escherichia coli* K99-specific monoclonal antibody. Infect. Immun. **42:**653–658.
22. **So, M., F. Heffron, and B. J. McCarthy.** 1979. The *E. coli* gene encoding heat stable toxin is a bacterial transposon flanked by inverted repeats of IS1. Nature (London) **277:**453–456.
23. **Stirm, S., F. Ørskov, I. Ørskov, and B. Mansa.** 1967. Episome-carried surface antigen K88 of *Escherichia coli*. II. Isolation and chemical analysis. J. Bacteriol. **93:**731–739.
24. **Westrom, B. R., J. Svendsen, and B. W. Korlson.** 1982. Protease inhibitor levels in porcine mammary secretions. Biol. Neonate **42:**185–194.
25. **Wolfe, K. H., P. L. Sadowski, H. J. Brandwein, A. Deutsch, and D. E. Reed.** 1984. A diagnostic kit for the detection of the K99 *E. coli* pilus in feces. Proc. Am. Assoc. Vet. Lab. Diagnost. **27:** 205–212.