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Occurrence of K99 Antigen on *Escherichia coli* Isolated from Pigs and Colonization of Pig Ileum by K99+ Enterotoxigenic *E. coli* from Calves and Pigs

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Several strains of enterotoxigenic *Escherichia coli* (ETEC) isolated from pigs were found to have an antigen (K99) previously reported only on strains of calf and lamb origin and which facilitates intestinal colonization in the latter two species. Several human ETEC were also tested for K99; however, none were positive. Each of four K99-positive ETEC strains of calf origin and one of pig origin produced K99 in pig ileum in vivo, adhered to villous epithelium in pig ileum, colonized pig ileum, and caused profuse diarrhea in newborn pigs. In contrast to the K99-positive strains above, four K99-negative ETEC from humans and chickens and one K99-positive ETEC from a calf either did not colonize pig ileum or did so inconsistently. When the K99-negative strains did colonize, they had little or no tendency to adhere to intestinal villi. These results are consistent with the hypothesis that K99 facilitates adhesion to and colonization of pig ileum by some ETEC.

Antigen K99 is common among enterotoxigenic *Escherichia coli* (ETEC) isolated from calves and lambs and facilitates colonization of calf and lamb small intestine (10, 13). Intestinal colonization by K99-positive ETEC appeared to be host species specific, in that such strains intensively colonized lamb but not pig small intestine and K99 was not reported among ETEC from other species. Host species specificity appears to be a general characteristic of ETEC in that calves, lambs, monkeys, and humans were not intensively colonized by pig ETEC fed to them (2, 11). Although some serotypes of *E. coli* have been isolated from cases of enteric disease in more than one host species, in general those isolated from different host species tend to be serologically different as well (14). Furthermore, K88 antigen, which is common among pig ETEC and facilitates colonization of pig small intestine, has not been reported among ETEC from other species. In contrast, there is evidence that some ETEC have the ability to colonize the small intestine of more than one host species. Evans et al. reported colonization of rabbit small intestine by an ETEC from humans (4), and Davidson and Hirsh (1) presented indirect evidence that K88-carrying ETEC of porcine origin colonize the small intestine of newborn mice.

The objectives of this manuscript are to report that: (i) some ETEC isolated from pigs have K99 antigen; (ii) some K99-positive ETEC isolated from calves have the ability to colonize the ileum and adhere to villous epithelium in pigs; and (iii) K99 antigen can be produced in pig ileum in vivo.

MATERIALS AND METHODS

***E. coli* strains.** The K99+ calf ETEC reference strains were B41, B44, and B117 (10). Additional K99+ calf ETEC used were strains 665, 1439, E90B, and 990A. The latter two were supplied by Steve Acres. Carlton Gyles supplied the laboratory strain K12 and a derivative of it that carried the K99 plasmid from strain B41 (K12-K99) and another derivative of it that carried a K88 plasmid (K12-K88). He also supplied the non-enterotoxigenic chicken strain F11 and a derivative of it carrying the enterotoxin plasmid from strain P155 [F11 (P155-Ent+)]. Sam Formal supplied human ETEC strains H10402, H13634, H10400, H10401, H10407, B7A, and B2C. The pig ETEC were the K88-negative strains 431, 613, 1351, 987, 340, 74-5208, and 381 described previously (9) plus TR75211 (Troyer) (8). In addition, 65 *E. coli* isolated from the intestine of newborn pigs with diarrhea were tested for K99 antigen. The pigs from which these isolates were taken came from several different herds in Minnesota, Iowa, Ohio,

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and South Dakota. Many, but not all, of the isolates had previously been shown to produce enterotoxin.

K99 antiserum. K99 antiserum was produced in rabbits according to the method for producing O:K antiserum (3). The antigen was strain K12-K99. The resulting hyperimmune serum was absorbed (3) with the parent K12 strain, which lacked the K99 plasmid. The resultant absorbed serum did not agglutinate the K12 parent but did agglutinate the K12-K99 used for immunization and the wild type, $K99^+$ calf ETEC reference strains. *E. coli* agglutinins in the absorbed serum were considered to be monospecific for K99. It was used in agglutination tests after a further 10-fold dilution and in indirect immunofluorescence tests after a further 100-fold dilution.

Demonstration of K99 on bacteria grown in vitro. This was done by slide agglutination tests using live bacteria. Bacteria were grown aerobically at 37°C (except when stated otherwise below) on Trypticase soy agar (BBL) or Minca agar (5) for 24 or 48 h. When available, individual small translucent colonies were selected as recommended (10). When small translucent colonies were not available, areas of confluent growth or individual colonies were selected at random for testing. Bacteria from a single colony or area of confluent growth were suspended in 2 separate drops of saline on a glass slide; K99 antiserum was added to 1 drop and normal rabbit serum was added to the other. All tests were replicated on 3 or 10 colonies or areas per plate. Suspensions that underwent grossly apparent agglutination in K99 antiserum and were not agglutinated in the normal rabbit antiserum were recorded as K99 positive.

Immunofluorescence tests for K99 were done by the indirect method, using K99 antiserum as the first antibody and fluorescein-labeled goat immunoglobulin G prepared against rabbit immunoglobulin G (Miles Laboratories, Inc.) as the second antibody. These were controlled by conducting parallel tests in which normal rabbit serum and/or K88 antiserum was (were) used as the first antibody on the same material tested for K99. The K88 antiserum was prepared the same way as K99 antiserum using strain K12-K88.

Cross agglutination and absorption. The methods for absorption of serum and production of 431-O:K antiserum (Table 1) were those of Edwards and Ewing (3). The antigens were grown on Minca for 24 h, except strain K12-K99, which was grown on Trypticase soy agar. To be sure that the colonies used for the tests were agglutinable in K99 antiserum, the tests were conducted such that portions of a single colony were tested in all four or five of the sera shown (Table 1).

Colonization of pig small intestine. Newborn hysterectomy-derived, colostrum-deprived pigs were exposed intragastrically to 1 ml of an overnight broth culture of *E. coli* as previously described (9). The pigs were killed 16 h postexposure, and observations on weight loss, numbers of *E. coli* per segment, and layers of *E. coli* adherent to villous epithelium in ileum were made as previously described (9). Pigs having $\geq 10^8$ viable test strain *E. coli* per segment were considered to be colonized. Each strain was tested in six pigs (two pigs from each of three different litters). The numbers of *E. coli* and the extent to which they were adherent to intestinal villi were determined for four pigs per strain. Layers of adherent *E. coli* were evaluated by the association index method (microscopic examination of immunofluorescent stained sections from colonized ileal segments) (9). Fluorescent antibody conjugates using the direct immunofluorescence method and antisera prepared against strains 431 and K12-K99 were both used to determine the association indexes of the K99-positive strains. Some sections were also examined by the indirect fluorescent antibody method. Antisera against the K99-negative strains were not available. Therefore, the association index for these strains was determined by using Giemsa-stained sections.

RESULTS

K99-positive pig ETEC. Four of the eight K88-negative pig ETEC initially tested were positive for K99. These were strains 431, 613, 1351, and Troyer, which had previously been shown to adhere to epithelium in and colonize pig ileum. Like some calf strains, K99 was

TABLE 1. *Results of slide agglutination tests using living E. coli antigens and rabbit sera as agglutinins*

Antigen			Antiserum				
Strain	Serotype	Origin	K99	431:O:K	K99 (absorbed by 431 antigen)	431-O:K (absorbed by K12-K99 antigen)	Unimmunized rabbit
B41	O101:K99:NM	Calf	+	+	0	NT[a]	0
B117	O8:K85,99:NM	Calf	+	+	0	0	0
665	O14:K99:NM[b]	Calf	+	+	0	0	0
K12-K99	K12:K99[c]	Laboratory	+	+	0	0	0
431	O101:K30(A):NM	Pig	+	+	0	NT	0
Troyer	O9:K35(A)	Pig	+	+	0	0	0

[a] NT, Not tested.

[b] It is not known whether or not strain 665 has a K antigen in addition to K99.

[c] The antigens of strain K12-K99 other than K99 are unknown and are indicated as K12.

sometimes difficult to demonstrate on these pig strains (5, 10) and results were not consistent from day to day. The percentage of positive reactions was increased by selecting small translucent colonies and growing on Minca rather than on Trypticase soy agar. For example, in one set of tests with strain 431, none of 10 small colonies from Trypticase soy agar were positive, whereas 2 of 10 large and 6 of 10 small colonies grown on Minca were positive. The numbers of positive colonies of the number tested for the Troyer strain in the same set of tests were: 3 of 10 for Trypticase soy agar; 3 of 10 for large and 7 of 10 for small colonies grown on Minca. Strains B41, 117, 431, and Troyer grown for 48 h on Minca were agglutinated by K99 antiserum when grown at 37°C but not when grown at 18°C.

There were no differences among the K99 antigens of the pig and calf strains tested by reciprocal cross agglutination and cross absorption tests (Table 1). Calf strains B41, B117, and 665, as well as pig strain Troyer and laboratory strain K12-K99, were all agglutinated in O:K antiserum to strain 431, demonstrating that the cross-reaction between K99 and strain 431 was two-way. Absorption of K99 antiserum with 431 antigen removed the K99 agglutinins. Furthermore, absorption of 431-O:K antiserum with K12-K99 antigen removed its B117, 665, and Troyer agglutinins. Strains 431 and Troyer were also examined at the WHO Collaborative Centre for Reference and Research on Escherichia, Copenhagen, Denmark, and their serotypes were found to be: O101:K30,K99:H$^-$ and O9:K35,K99:H$^-$.

Some of the 65 additional *E. coli* isolates from the intestines of newborn pigs with diarrhea also carried K99. At least one agglutinable colony was detected in 10 of 18 isolates of serogroup O101:K30(A), in 4 of 4 isolates of O64, in 0 of 8 isolates of O101:K+(A), in 0 of 18 isolates of O9, and in 0 of 17 isolates of O20. The human ETEC strains were all negative.

Colonization of pig small intestine by calf ETEC. Five K99-positive bovine strains and five K99-negative strains of chicken or human origin were tested in parallel (Table 2). Four of the five calf strains (B44, E90B, 665, and 1439) consistently colonized pig ileum (more than 10^8 *E. coli*/segment) and had high association indexes (layers of *E. coli* adherent to villous epithelium) (Table 2, Fig. 1). The adherent *E. coli* stained with both fluorescent antisera (anti-K12-K99 and anti-431), and both antisera gave the same association indexes. The colonizing and adhesive attributes of these four strains were comparable to those for pig enteropathogens tested in this system previously (9). The incidence of diarrhea and severity of weight loss caused by these four strains was also comparable to that caused by pig enteropathogens (9). Two of the pigs exposed to strain E90B did not develop diarrhea; unfortunately, the numbers of *E. coli* and the association indexes for these two pigs were not determined.

In contrast to the other four calf K99-positive ETEC, strain 990A did not colonize the ileum of any of the pigs, nor did the enterotoxigenic chicken strain F11 (P155-Ent$^+$) or human strain B7A. Chicken strain F11 and human strains H10407 and B2C colonized (attained more than

TABLE 2. *Diarrhea and intestinal colonization of newborn pigs exposed to E. coli from different sources*

E. coli inoculum			Pigs 16 h postexposure				
					Ileal *E. coli*[a]		
Strain	Source	Serotype	Diarrhea	% Wt loss (mean)	No.[b]		Association index (mean)[c]
					Mean	Range	
B44	Calf	O9:K30,99:NM	6/6	17	7×10^8	10^8–10^9	4.5
E90B	Calf	K99[d]	4/6	14	2×10^9	10^9	4.8
665	Calf	O14:K99[d]	6/6	20	2×10^9	10^8–10^9	4.4
1439	Calf	K99[d]	6/6	19	1×10^9	10^8–10^9	4.4
990A	Calf	O8:K85,99:H27	4/6	9	9×10^6	10^5–10^7	
H10407	Human	O78:K80:H11	5/6	6	1×10^8	10^7–10^8	1.0
B7A	Human	O148:H25[d]	6/6	13	2×10^6	10^6	
B2C	Human	O6:H16[d]	6/6	9	2×10^8	$<10^5$–10^8	1.0
F11(P155-Ent$^+$)	Chicken	?[d]	3/6	8	5×10^6	10^5–10^7	
F11	Chicken	?[d]	0/6	4	7×10^7	10^7–10^8	2.0

[a] Four pigs per strain.
[b] Number of viable test strain *E. coli* per 10-cm segment of ileum.
[c] Microscopic evaluation of intensity of adhesion to intestinal villi in pigs with $\geq 10^8$ *E. coli* per ileal segment (5.0 is maximal, 1.0 is minimal).
[d] Complete serotype unknown.

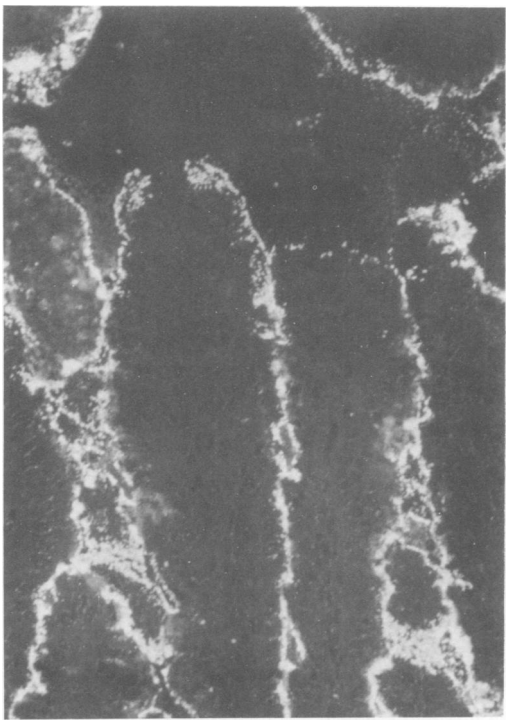

FIG. 1. *E. coli of K99-positive calf strain B44 adherent to villous epithelium in ileum of newborn pig (association index, 5.0). Frozen section stained directly with fluorescent antibody prepared against E. coli strain 431.*

10^8/segment) 5 of the 12 pigs examined bacteriologically. However, even in these five colonized segments, these strains had little or no tendency to adhere to epithelium (association indexes, 2 and 1; Table 2).

Diarrhea occurred in 22 of 24 (92%) pigs exposed to the four consistently colonizing strains (Table 2), and the mean weight loss of these 24 pigs was 17.5%. Diarrhea occurred in 24 of 30 (80%) pigs exposed to the five noncolonizing or inconsistently colonizing ETEC, and these 30 had a mean weight loss of 9%. None of the pigs exposed to the non-enterotoxigenic strain F11 developed diarrhea, and these had a mean weight loss of 4%.

In vivo production of K99. Presumptive evidence that calf strains can produce K99 in pig ileum was provided by the observation that layers of strains B44, E90B, 665, and 1439, adherent to ileal villi in pigs 16 h postexposure, consistently stained with fluorescent antibody prepared against strain K12-K99 and strain 431 (Table 2, Fig. 1). The only antigen known to be common to all six of these strains is K99. Sections from the ilea of pigs exposed to the four calf strains above were recut and stained with the K99 antiserum (from which K12 antibodies had been removed by absorption) via the indirect immunofluorescent method, using normal rabbit serum as the control. All sections exposed to K99 antiserum were positive (cf. Fig. 1), and those exposed to normal rabbit serum were negative (the adherent layers of bacteria were not stained).

Sections of ileum from three pigs used in previous experiments (9) and shown to have layers of strain 431 adherent to their villi were recut (three slides per pig) and examined by the indirect immunofluorescence method, using K99 antiserum as the first antibody for one slide, K88 antiserum as the first antibody for the second slide, and normal rabbit serum as the first antibody for the third slide from each pig. Layers of strain 431 adherent to villi were demonstrable in all three sections exposed to K99 antiserum (Fig. 2, 3). These were not visible in sections exposed to K88 antiserum or normal rabbit serum.

DISCUSSION

The pig ETEC strains 431 and Troyer were shown by serum agglutination and cross absorption tests to have an antigen either closely related or identical to the K99 antigen of a K12 strain to which the K99 plasmid from a calf strain had been transferred. Like K99 antigen, that on the pig ETEC was also: (i) more readily demonstrable in small than in large colonial forms, (ii) more readily demonstrable in colonies grown on Minca than in those grown on Trypticase soy agar, and (iii) suppressed when grown at 18°C (10). We interpret these results to mean that the antigen on the pig ETEC is identical to K99. In view of the preliminary survey conducted, it seems probable that K99 occurs rather commonly on pig strains of O groups 101 and 64 and occasionally on those in O group 9 and may occur in other O groups as well. Our failure to demonstrate it on human ETEC could be because it is not carried by human ETEC. Alternatively, it may be carried by human ETEC other than those tested, or it could have been present on the human ETEC tested but not detected. The latter possibility warrants consideration in view of the difficulty in consistently demonstrating this antigen on some strains.

Presumably the K99 demonstrated in vivo was produced in vivo as the inocula contained approximately 10^9 cells and the segments examined for K99 contained 10^8 to 10^9/10 cm by 16 h postexposure. There is evidence that K99 fa-

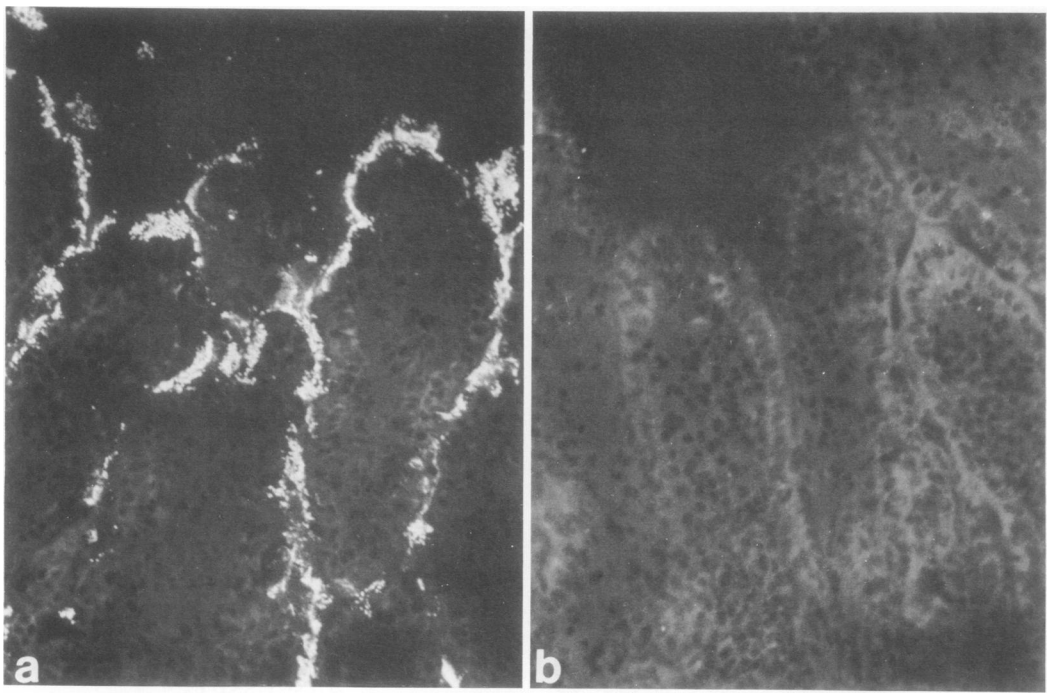

FIG. 2. *K99 antigen produced by pig E. coli strain 431 in pig ileum in vivo. (a) Layers of bacteria adherent to villi were stained for K99 by the indirect immunofluorescent method using specific K99 antiserum. (b) Section from same segment of ileum as (a) but stained via specific K88 antiserum. The K99-positive bacteria are not stained. Indirect immunofluorescence.*

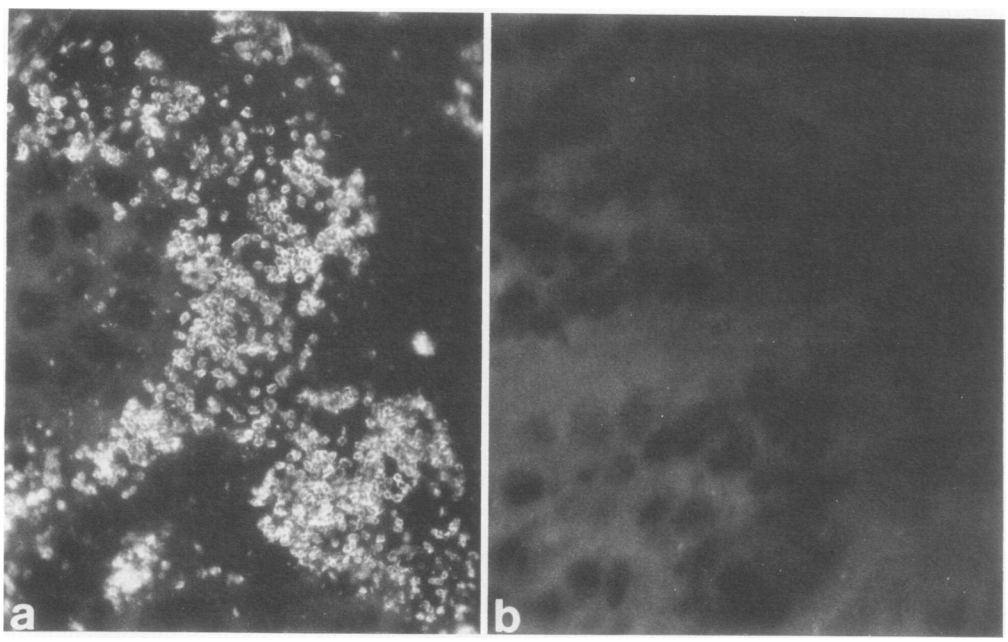

FIG. 3. *K99 antigen produced by pig E. coli strain 431 in pig ileum in vivo, as Fig. 2, except for (a) higher magnification to demonstrate that the antigen occurs as a ring of peribacterial fluorescence. (b) Section from same segment of ileum as (a) but stained with K88 antiserum. The K99-positive bacteria are not stained. Indirect immunofluorescence.*

cilitates colonization of calf and lamb small intestine, and it was suggested that K99 might act as an adhesin to facilitate such colonization (13). Studies of adhesion by K99-positive strains have not previously been reported. The data reported here demonstrate that K99-positive ETEC can adhere in pig ileum. However, they do not permit conclusions as to whether or not the adhesion was mediated by K99. In contrast to the results reported here, K99-positive calf and lamb ETEC apparently did not colonize pig small intestine (13). The reasons for these different results between laboratories, using different strains and different pigs, are unknown. Strain 990A did not colonize pigs in this study, demonstrating that not all K99-positive ETEC colonize pig ileum in the model used here. Furthermore (even though present), K99 may not have facilitated colonization of pig ileum by the other four strains, which were consistent colonizers (Table 2). That is, the latter strains may have had attributes other than K99 that allowed them to adhere to epithelium and colonize pig ileum. This would imply that such strains possess K99, which facilitates colonization of calf and lamb small intestine, and an additional attribute(s) that facilitates colonization of ileum in the newborn pig. However, both pig and calf ETEC produced K99 in pig ileum in vivo. For this reason we are more attracted to the hypothesis that the ileum of the newborn pig provides an environment comparable to calf intestine with regard to colonization by ETEC and that K99 facilitates colonization in this environment. Assuming this hypothesis is correct, the K99 of the noncolonizing strain 990A, as used here, could be quantitatively or qualitatively defective, or the remainder of the bacterial cell could lack other unknown characteristics necessary for colonization.

Pigs exposed to ETEC developed diarrhea even though not colonized (less than 10^8 *E. coli*/10 cm of ileum). These pigs may have had more than 10^8 *E. coli*/10 cm at other sites in the small intestine. Alternatively, fewer than 10^8 ETEC/10 cm may be sufficient to cause diarrhea. Weight loss of pigs colonized by ETEC exceeded that of those exposed to but not consistently colonized by ETEC (Table 2). This indicates that diarrhea was more profuse in the colonized pigs and is additional evidence that the consistent colonizers were more virulent than the inconsistent and noncolonizing ETEC.

K88 antigen is a pilus, or pilus-like, structure (15), which is produced by many pig ETEC and facilitates adhesion to and colonization of pig small intestine (7, 12). There is evidence that pili such as those on pig ETEC strain 987 (distinct from K88 and K99 and designated as 987-P) also facilitate adhesion and colonization in pig small intestine (R. E. Isaacson, B. Nagy, and H. W. Moon, J. Infect. Dis., in press). There is evidence that K99 is also a pilus (6). These observations and the data reported here are consistent with the hypothesis that K99 is one of several pili occurring among pig ETEC that act as adhesins to facilitate intestinal colonization. However, before acceptance of the hypothesis, the roles of K99 and 987-P, both in adhesion and in colonization of pig small intestine, should be investigated directly. Information as to how many and which pili of ETEC are involved in intestinal colonization and their host species specificity will have useful diagnostic, epidemiological, and preventative implications for enteric infections with ETEC.

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