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Type 1 Pili (F1) of Porcine Enterotoxigenic *Escherichia coli*: Vaccine Trial and Tests for Production in the Small Intestine During Disease

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This study was performed to determine whether the F1 (type 1) pili of a porcine strain of enterotoxigenic *Escherichia coli* are protective antigens and whether they are produced in the pig small intestine during disease caused by an enterotoxigenic *E. coli*. Reciprocal cross-absorption experiments with antisera prepared against F1 pili purified from enterotoxigenic *E. coli* 431 (O101:K30,99:H^-:F1) and P14 (O149:K91,88ac:H^+:F1) demonstrated that the F1 antigens of the two strains were closely related or identical. Pregnant swine vaccinated with a vaccine prepared from strain P14 ($F1^+$) responded with a significant increase in antibody against F1 in their serum and colostrum. However, the vaccinated dams did not significantly protect their suckling pigs against fatal challenge with strain 431. There was no evidence of F1 pilus production in the strain 431-infected pigs, as determined by immunofluorescent staining of ileal sections, direct electron microscopic examination of bacteria from ilea, and titration of serum agglutinins in convalescent pigs. It was concluded that strain 431 did not produce F1 in the small intestine during disease and that F1 was not a protective antigen in this system.

Pili (fimbriae) of several antigenic types facilitate adhesion to the mucosa and thus colonization of the small intestine by enterotoxigenic *Escherichia coli* (ETEC). Pili of antigen types K88 (F4; see reference 23 for a discussion of antigen nomenclature), K99 (F5), and 987P (F6) facilitate intestinal colonization in swine, and pili of antigen types CFAI (F2) and CFAII (F3) facilitate intestinal colonization in humans (reviewed in references 8 and 18). It seems probable that additional antigenic types of pili which permit intestinal colonization by ETEC in animals (1, 4, 21; S. C.-M. To, submitted for publication) and humans (12, 13; M. M. Levine, R. E. Black, M. L. Clements, C. R. Young, C. C. Brinton, P. Fusco, S. Wood, E. C. Boedeker, C. Cheney, P. Schadl, and H. Collins, *in* E. C. Boedeker, ed., *Attachment of Microorganisms to the Gastrointestinal Mucosal Surface*, in press) will be found. When pregnant swine are vaccinated with F4, F5, or F6 antigens, their suckling newborn pigs are protected against challenge with ETEC of homologous, but not heterologous pilus antigen types (20, 22, 27, 28; reviewed in reference 18). These antigens are attractive candidates for practical vaccines because they (i) are on the bacterial surface and thus readily accessible to antibody, (ii) are produced in the intestine as a critical step early in the pathogenesis of the disease, and (iii) permit broad protection with vaccines of restricted antigenic valence (F4, F5, and F6 for swine and F5 for cattle and sheep).

Pili of the above-mentioned antigenic types tend to be restricted to ETEC (ETEC specific) and to ETEC from a single or narrow range of host species (8). In contrast, F1 pili (type 1 or common pili; see reference 23) are produced by most pathogenic and nonpathogenic *E. coli* from a wide variety of host species (2, 5, 23). Although many ETEC produce F1 in addition to ETEC-specific pili (3, 7, 23; To, submitted for publication) and F1 pili facilitate adhesion of *E. coli* to intestinal epithelial cells in vitro (9), it is not known whether F1 pili (i) are produced in the small intestine, (ii) facilitate colonization of the small intestine, or (iii) are capable of serving as protective antigens during ETEC infection. We think that protection induced by ETEC-specific pilus antigens depends on antibody which blocks adhesion of pili to specific epithelial receptors. However, it is conceivable that protection could also be the result of agglutination, steric hindrance, altered bacterial surface charge, or opsonization by antibodies directed against ETEC-specific pili. It is also possible that any surface antigen produced by ETEC during colonization of the small intestine might provide protection by one of the (or some other) mechanisms. Thus, if F1 is produced in the small intestine, it might be a protective antigen even if it does not facilitate colonization.

The primary objective of the experiments reported here was to detemine whether swine vaccinated with a strain of F1-bearing *E. coli* would protect their suckling newborn pigs against challenge with a heterologous F1-producing ETEC. The secondary objective was to determine whether the challenge strain produces F1 in the small intestines of pigs during disease. We found that vaccination with F1 did not protect the newborns, and we were unable to demonstrate production of F1 in their small intestines.

MATERIALS AND METHODS

***E. coli* strains and growth conditions.** The *E. coli* strains used in this study are listed in Table 1. Strain P14, which produces F1 and F4 pilus antigens and heat-labile enterotoxin, was used as the vaccine strain. Strain 431, which produces F1 and F5 pilus antigens and heat-stable enterotoxin demonstrable in infant mice, but not heat-labile enterotoxin, was used as the challenge strain. Strain 431 was grown in Trypticase soy broth (TSB; BBL Microbiology Systems, Cockeysville, Md.) and stored at −50°C until just before it was administered to the pigs (10^{10} viable bacteria per pig by gavage) as reported previously (15). This dose was used because in previous experiments a similar inoculum (10^{10} strain 431 organisms per pig) caused death in 50 to 74% of control pigs from this herd by day 5 after inoculation (14, 15; H. W. Moon and P. L. Runnels, *in* E. C. Boedeker,

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TABLE 1. *E. coli* strains used[a]

Strain	Serotype	Application[b]	Reference or source[c]
P14	O149:K91, 88ac:H[+d]	Vaccine, T, A	PSU
D616	O149:K91,88⁻:H10	T	25; K88⁻ of strain CS1483
431	O101:K30,99:H⁻	Challenge, T, A	19
1793	O9:K30	T	NVSL
1476	K12(K88)	T	C. L. Gyles
Troyer	O9:K35, 99:H⁻	T	E. M. Kohler
E990A	O8:K85,99	T	S. D. Acres
G7	O8:K87,88:H19	T	PSU

[a] All of these strains have been demonstrated to be F1 producers (S. C. To, unpublished observations).
[b] T, Titration antigen; A, absorbing antigen.
[c] PSU, Pennsylvania State University, University Park; NVSL, National Veterinary Services Laboratory, Ames, Iowa.
[d] Strain motile but untypable.

Attachment of Microorganisms to the Gasrointestinal Mucosa Surface, in press).

Methods for selecting type 1 (F1) phase variants ($F1^+$) from ETEC strains have been described previously (29; To, submitted for publication). Bacteria were streaked onto Trypticase soy agar (BBL Microbiology Systems) and incubated at 37°C for 16 to 20 h. $F1^+$ colonies, recognized as medium to small with raised contour, and $F1^-$ colonies, recognizd as medium to large with flat contour, were selected as previously described (To, submitted for publication). The colonies were then subcultured individually in TSB and incubated at 37°C with vigorous aeration for 6 to 8 h. $F1^+$ or $F1^-$ characteristics of cells from these cultures were verified by electon microscopy and/or characteristic agglutination with monospecific rabbit serum prepared against purified F1 pili (3). Those cultures verified as $F1^+$ were pooled, and samples were stored in sealed vials at −60°C to be used as primary seeds for pilus purification and preparation of test antigen, absorbing antigen, or vaccine.

Vaccine production. A primary seed suspension of *E. coli* P14 ($F1^+$) was thawed and subcultured (1:100) into TSB and incubated at 37°C with vigorous aeration for 6 h. The $F1^+$ characteristics of the culture was verified by agglutination with monospecific F1 antiserum. The culture was used to seed (1:400) a fermentor containing 20 liters of TSB. The fermentor culture was incubated at 37°C with vigorous aeration for about 7 h, while growth of the culture was monitored photometrically at 540 nm, and the $F1^+$ characteristics of the cells were verified by agglutination with specific serum. When the culture was grown to an estimated 5×10^9 viable cells per ml, Formalin was added to a final concentration of 0.3% (vol/vol). Inactivation was continued for 48 h at room temperature, at which time 0.3% (wt/vol) aluminum hydroxide was added to the vaccine as an adjuvant, and the suspension was mixed at 4°C for 24 h.

Vaccination schedule. Gilts in the vaccinated group were each given two 2-ml doses of the vaccine intramuscularly behind the ear. The first dose was given 1 month before anticipated farrowing, and the second dose was given 2 weeks later. Gilts in the control group were not vaccinated. In preliminary trials with this vaccine and vaccination schedule, 1 of 68 pigs born to and suckling vaccinated dams and 14 of 41 pigs in an unvaccinated control group died of diarrhea by day 5 after challenge with the vaccine strain P14 (10^9 viable *E. coli* per pig). In contrast, 9 of 21 pigs in the vaccinated group and 11 of 21 pigs in the unvaccinated control group died of diarrhea by day 5 after challenge with *E. coli* 431 (10^9 viable *E. coli* per pig).

Antiserum preparation and serological methods. Rabbit hyperimmune sera were prepared with F1 pilus antigen purified from strains P14 ($F1^+$) and 431 ($F1^+$). Frozen samples of the appropriate $F1^+$ forms were thawed, subcultured (1:100) in TSB, and incubated at 37°C with vigorous aeration for 5 to 6 h. These broth cultures were used to seed Trypticase soy agar (BBL) contained in rectangular glass baking dishes. After 16 to 20 h of growth at 37°C, the cells were harvested into 0.05 M morpholinepropanesulfonic acid buffer (pH 7.0; Sigma Chemical Co., St. Louis, Mo.). The suspensions were kept cold in an ice-bath and sheared at top speed (16,000 rpm) in an Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.) operated in intermittent bursts for 4 min. After centrifugal clarification, F1 pili were purified from supernatant by five cycles of magnesium chloride crystallization, and a UV spectrum characteristic of the F1 pilus (3) was obtained. The soluble form of such purified antigen was used to immunize rabbits by standard methods for hyperimmune serum (6).

Blood was taken from vaccinated gilts just before the first vaccination and 2 weeks after the second vaccination and from control gilts during the last week of gestation. Colostrum was collected from all gilts at parturition. Blood was taken from 19 18-day-old pigs born to unvaccinated dams.

Serum and colostral whey were titrated from F1 agglutinin, using formalinized (0.5% [vol/vol]) F1-bearing *E. coli* as test antigen. To minimize nonspecific agglutination, test antigens were produced from strains of serotypes heterologous to the strains to which the animals were exposed (through vaccination or challenge infection). Appropriate $F1^+$ primary seeds were subcultured in TSB as described earlier, and formalinized cultures used as test antigens were standardized photometrically at 540 nm to obtain an optical density of 0.5. Agglutinin titers were determined in microtiter plates with 96 U-shaped wells. The test plates were incubated for 2 h at 37°C, followed by overnight incubation at 4°C. Negative as well as positive reference sera were included in each test. The agglutinin titer was expressed as the reciprocal of the highest dilution of the test sample exhibiting distinct agglutination of the test antigen.

Indirect immunofluorescence tests for F1, F4, and F5 pilus antigens in layers of strain 431 adherent to pig ileal mucosa were performed by procedures reported previously (17). Reference absorbed monospecific K99 (F5) and K88 (F4) antisera were used as previously reported (17). Hyperimmune rabbit antiserum prepared against purified F1 (P14) was used to test for F1. This latter antiserum agglutinated the $F1^+$ but not the $F1^-$ form of strain 431 and gave positive results in indirect immunofluorescence tests when tested against in vitro-grown $F1^+$ bacteria of strain 431 smeared on a glass slide and fixed in methanol.

Electron microscopy. Ileal fluid harvested directly from three diarrheal pigs was examined electron microscopically for bacteria bearing structures resembling F1 pili. Negatively

TABLE 2. Relative agglutinin titers against *E. coli* antigens in sera raised with purified F1 (type 1) pilus antigens from *E. coli* P14 and 431

Antiserum	Relative agglutinin titers against antigen[a]							
	P14 (F1+)	O149	K91	K88	431 (F1+)	O101	K30	K99
F1 (P14)	1	0.003	0	0.006	0.25	0	0	0
F1 (431)	4	0	0	0	1	0.003	0	<0.001
F1 (P14) abs/431[b]	0	0.002	0	0.001	0	0	0	0
F1 (431) abs/P14	0	0	0	0	0	<0.001	0	<0.001

[a] Homologous reactions were assigned a value of 1, and other reactions were assigned proportional values. O antigens were prepared by standard methods (6), strain D616 selected for F1$^-$ was used as the K91 antigen, strain 1793 selected for F1$^-$ was used as the K30 antigen, strain 1476(K12-K88) was used as the K88 antigen, and strain Troyer was used as the K99 antigen. The antigens were formalinized and standardized as described in the text. Standard reference antisera against these antigens were used as positive controls.

[b] See text for method of cross absorption.

stained preparations were made and examined by a procedure reported previously (10). For shadow-cast preparations, ileal fluid samples were mixed 1 to 1 with 1% Formalin in 0.85% saline. Specimens were prepared by agar filtration (11) and shadowed with platinum.

Animals. Pregnant gilts were placed in isolation rooms 1 month before anticipated parturition. Pigs born to the gilts were allowed to suckle at will, and all but two litters (unchallenged controls) were challenged with strain 431 before they were 7 h old.

Observations. Procedures used to evaluate the response of pigs to challenge were as reported previously (15). Pigs were examined clinically on days 1 and 5 after challenge. Deaths due to diarrhea and body weight of survivors were recorded until day 5 after challenge. On day 1 after challenge a pig from each litter was picked randomly, killed, and necropsied to provide intestinal sections for histological and bacteriological evaluation.

Statistical analyses. Percent weight change and agglutinin titers between groups of pigs were analyzed by Student's t test for two means. Diarrhea incidence and mortality rate were evaluated by a 2 × 2 contingency table with Yate's correction for continuity.

RESULTS

Antigenic studies of F1 pili on strains 431 and P14. The hyperimmune antisera prepared against F1 antigens from strains 431 and P14 were titrated by the microtiter plate method for agglutinins against the *E. coli* antigens listed in Table 2. The data in Table 2 show that the F1 pilus preparations used for serum production elicited only minor agglutinating activities against some other antigens, such as O149 and K88 (F4) for strain P14 and O101 for strain 431, when compared with the high homologous anti-F1 titers.

When antisera against F1 prepared from strains 431 and P14 were each repeatedly (six times) cross-absorbed with their heterologous F1$^+$ form, all anti-F1 activities were removed (Table 2).

Serological response of vaccinated gilts. Serum and colostral whey from nonvaccinated control gilts and prevaccinal serum from vaccinated gilts had low titers of agglutinins for the F1$^+$ form of strain 431 (Table 3). F1 (strain 431) agglutinin titers in serum and colostrum from vaccinates after vaccination with strain P14 (F1$^+$) were significantly ($P < 0.01$) higher than those from unvaccinated control gilts (Table 3). The validity of the F1 agglutinin determination was verified by duplicate titrations of these samples against another test antigen, *E. coli* G7 (serotype O8:K87,88:H19), selected for the F1$^+$ characteristic (data not shown). The magnitude and statistical significance of the difference in F1 agglutinin titers between samples from vaccinated and unvaccinated gilts were similar whether determined with strain 431 (F1$^+$) or strain G7 (F1$^+$).

Response of pigs to challenge with *E. coli* 431. Two litters of pigs born to unvaccinated control gilts were left as unchallenged controls. These 17 pigs remained normal and had a mean increase of 57% in body weight from birth to day 5 (Table 4).

In contrast, there was a high incidence of fatal diarrhea among pigs challenged with strain 431 in both the control (three litters) and vaccinated (five litters) groups (Table 4). Some survivors still had diarrhea on day 5. Challenged pigs in both groups were intensively colonized by *E. coli* with layers of bacteria adherent to their intestinal villi. Survivors in both groups either gained very little or lost weight during the period of observation. Statistical analyses showed no significant difference between the control and vaccinated groups in mortality rate or percent weight change among the survivors, but the prevalence of diarrhea at day 5 was significantly greater ($P < 0.01$) in controls.

Production of F1 pili in small intestine. Frozen sections of the ileum were prepared from five pigs from the vaccine group and from one control pig the day after challenge with strain 431. These sections were stained with F5, F4, and F1 (P14) antisera by indirect immunofluorescence. All sections had layers of bacteria adherent to the villi. These layers stained with F5 but not with F4 or F1 (P14) antisera.

Ileal fluids from three control pigs killed the day after challenge with strain 431 were prepared for direct electron microscopic examination both as negativly stained and as shadow-cast preparations. A total of 207 bacterial rods resembling *E. coli* were examined in the negatively strained preparations, and 5 had structures resembling F1 pili. A total

TABLE 3. Effect of vaccinating pregnant swine with ETEC P14 (O149:K91,88ac:H$^+$:type 1 pili[a]) on the geometric mean titers of agglutinin against ETEC 431 (O101:K30,99:H$^-$:type 1 pili[a]) In serum and colostrum

Gilts		Titers in[b]:		
		Serum		
Vaccine	No.	Prevaccination	Postvaccination	Colostrum
None	5		7 (4–8)	18 (16–32)
P14 (F1)	5	21 (2–256)	170[c] (8–512)	339[d] (32–512)

[a] Type 1 pili are also designated F1.

[b] Titers are geometric means of reciprocals of highest dilutions causing agglutination. Ranges are given in parentheses.

[c] Significantly different from nonvaccinated group ($P < 0.01$) and from those before vaccination ($P < 0.05$).

[d] Significantly different from nonvaccinated group ($P < 0.01$).

TABLE 4. Effect of vaccinating pregnant swine with ETEC P14 (O149:K91,88ac:H$^+$:type 1 pili[a]) on the response of their newborn suckling pigs to challenge with ETEC 431 (O101:K30,99:H$^-$:type 1 pili[a])

Newborn pigs			Clinical signs (%)				*E. coli* present at necropsy on day 1 in[b]:					
Vaccine group	Challenge strain	No.	Diarrhea		Weight change[c]	Died[d]	Jejunum (upper)		Jejunum (lower)		Ileum	
			Day 1	Day 5			No.[e]	Adherent[f]	No.[e]	Adherent[f]	No.[e]	Adherent[f]
Control	None	17	0	0	+57	0	7.0	0/2	7.0	0/2	7.9	0/2
Control	431	26	100	86	−8	70	6.8	1/3	9.6	3/3	9.6	3/3
P14 (F1$^+$)	431	45	100	6	+6	58	6.9	1/5	8.7	5/5	9.5	5/5

[a] Type 1 pili also designated F1.
[b] One pig per liter was killed and necropsied 1 day after challenge.
[c] Mean change from initial (day 0) body weight in pigs surviving to day 5.
[d] Died of diarrheal disease by day 5.
[e] Mean $\log_{10}$ number of viable *E. coli* per 10 cm of intestine.
[f] Number of pigs with layers of bacteria adhering to villi per number of pigs examined.

of 146 bacterial rods resembling *E. coli* were examined in the shadow-cast preparations. None had structures resembling F1 pili.

Fifteen pigs from unchallenged control litters and four pigs from challenged control litters were reared in isolation for 18 days after challenge. They were then bled to determine titers of serum agglutinins against F5 and F1. A formalinized (0.5%) culture of *E. coli* Troyer, which agglutinated in F5 antiserum but not in F1 pilus antiserum, was used as the antigen for F5 agglutinin titers, and a formalinized culture of P14 (F1$^+$) which agglutinated in reference F1 antiserum was used as the antigen for F1 agglutinin titers. Agglutinin titers of <1:4 (the lowest dilution tested) were recorded as 0. The F5 (K99) agglutinin titers of pigs that survived challenge with strain 431 (mean, 14; range, 8 to 16) were significantly higher than those of pigs that were not challenged (mean, 2; range, 0 to 16). In contrast, the F1 (type 1) pilus agglutinin titers of pigs that survived challenge with strain 431 (mean, 88; range 32 to 128) were not higher than those of pigs that were not challenged (mean, 124; range, 64 to 256). The validity of F5 (K99) and F1 agglutinin titration was confirmed by duplicate tests of these serum samples, using test antigens prepared from *E. coli* E990A and G7, selected for F5 and F1, respectively.

DISCUSSION

The preliminary experiments with the P14 (F1$^+$) vaccine and homologous challenge demonstrated that the vaccine and vaccination schedule used were adequate to protect newborn pigs by lactogenic immunity. Such homologous protection in the preliminary experiments was probably due to antibody against K88 pili, heat-labile enterotoxin, somatic antigens, or a combination of these. In contrast, vaccination did not protect against heterologous challenge by *E. coli* 431 in the preliminary experiments.

The antigenic analysis of F1 antigens purified from strains P14 and 431 (Table 2) demonstrated that the antigens are closely related or identical. The increased titers of 431 (F1$^+$) agglutinins in serum and colostrum after vaccination with P14-F1$^+$ in the experiment reported here (Table 3) were attributed to antibodies against F1 because, insofar as we can tell, that is the only surface antigen common to strains 431 and P14. Nevertheless, the vaccinated gilts did not protect their suckling pigs against intensive intestinal colonization and fatal diarrhea caused by strain 431 (Table 4). We interpret these data to demonstrate that F1 is not a protective antigen for ETEC strain 431 in this system.

The prevalence of diarrhea among survivors at day 5 (Table 4) was significantly less in the vaccine group than in the challenged control group. This difference could be the result of low-level vaccine-induced protection due to F1 or some unrecognized antigen common to strains P14 and 431. However, it seems more likely to be due to variation among litters unrelated to vaccination. In previous experiments with the challenge used here (10^{10} strain 431 organisms per pig) the prevalence of diarrhea among surviving control pigs from this herd at day 5 varied from 10 to 43% (14, 15; Moon and Runnels, in press).

Three lines of evidence indicate that strain 431 produced little or no F1 antigen in the small intestine of the pigs during the disease. First, layers of strain 431 on ileal villi in sections from diseased pigs stained with F5 but not F1 antiserum by immunofluorescence. Second, pigs recovered from challenge did not have higher titers of serum agglutinins against F1 pilus antigen than did unchallenged control pigs, whereas they did have higher titers against F5 antigen. Third, most bacteria in ileal fluids from diseased pigs lacked F1 (type 1) pili, demonstrable by direct electron microscopic examination of whole mount preparations. F6 (987P) pili, which are morphologically similar to F1 pili, can be readily demonstrated in intestinal fluids of diseased pigs by the latter technique (10). Pig small intestine provides strong selection or promotion of F5 and F6 (16), whereas there appears to be little or no production of F1 pili by strain 431 in this environment. Attempts to demonstrate F1 production in the pig small intestine by other strains of porcine ETEC were also unsuccessful (1).

Our negative results regarding F1 production in the small intestine by porcine ETEC and F1 as a protective antigen via passive lactogenic immunity in pigs are similar to those of Levine et al., who studied active immunization in humans (12; Levine et al., in press). They found that humans did not have increased serum antibody titers against F1 after recovery from ETEC infection (although they did have increased titers against F2, enterotoxin and somatic antigen). Furthermore, they were not able to protect themselves against intensive intestinal colonization and disease by an active immunization regimen which stimulated production of increased level of immunoglobulin A against F1 in the lumen of the small intestine. Results reported here tend to confirm their results and to generalize the conclusion by extension to another host species, with another ETEC strain, and another antibody delivery system (passive lactogenic immunity). We think that F1 pili are not protective antigens for ETEC infections because, in contrast to ETEC-specific pili, F1 pili are not produced in the small intestine during disease.

Normal humans and animals usually have antibody against F1 (12; Levine et al., in press; S. C.-M. To, unpublished data), which presumably indicates F1 antigen production in vivo. There is serological evidence of F1 antigen production in vivo during urinary tract infections with *E. coli* (26). The data are consistent with the hypothesis that F1 pili are produced in the urinary tract and in the natural habitat of *E. coli*, the large intestine (24).

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