Effect of Oral Egg Antibody in Experimental F18$^+$ *Escherichia coli* Infection in Weaned Pigs

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ABSTRACT. The protection conferred by egg antibody specific for F18-fimbriae against infection with F18$^+$ *Escherichia coli* was studied in controlled passive immunization trials involving weaned pigs. Parameters of protection consisted of body weight gain, frequency and severity of diarrhea and recovery of the challenge strain of F18$^+$ *E. coli*. Weaned pigs at four weeks of age were challenge exposed once daily for three days by oral inoculation with 10^{11} cfu of virulent F18$^+$ *E. coli* followed by daily administration of antibody supplemented feed for 9 days starting from the first challenge day 0. Results showed a dose-dependent response to antibody treatment. The group of pigs given 1:50 titer of antibody in feed had less frequency of diarrhea ($P<0.01$–0.05), higher rate of gain ($P<0.01$) and lower isolation rate of challenge strain in rectal and intestinal swabs ($P<0.01$) compared to non-treated control. In the same manner, the anti-F18 antibody significantly reduced adherence of F18$^+$ *E. coli* to pig intestinal epithelial cells *in vitro* ($P<0.01$). Results suggest that egg antibodies specific for the F18 fimbriae is a suitable immunotherapeutic agent for pigs infected with F18$^+$ *E. coli* and that pigs can be protected from overt clinical disease and the subsequent reduced performance arising from infection with this pathogen. — KEY WORDS: egg antibody, *Escherichia coli*, F18 fimbriae, passive immunity, porcine postweaning diarrhea.

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Post-weaning colibacillosis is a major cause of economic loss in the pig industry arising from increased mortality and reduced performance. Enterotoxigenic *E. coli* have been associated with diarrhea in neonatal and postweaned piglets and its fimbriae have been recognized as colonization factors [7]. Aside from colonization factors K88, K99, and 987P which have been associated with neonatal enteric colibacillosis, the recently characterized colonization factors F18ab (formerly F107) [1] and F18ac (formerly 2134P, Av24, 8199, or 8813) [4, 8–10] have also been associated with postweaning diarrhea and edema disease in pigs. In a study in Switzerland [9], the relatedness of the fimbriae F18ab and F18ac was confirmed by polymerase chain reaction (PCR) and serological methods (nomenclature used was F107 for F18ab and 2134P or 8813 for F18ac). Independent studies in Australia [2] and Sweden [5] showed a prevalence rate of 62% and 30% based on hybridization test using F18 specific oligonucleotide probes thereby illustrating the epidemiological importance of this *E. coli* phenotype in pigs.

Control of diarrhea caused by enterotoxigenic *E. coli* have so far been largely based on anti-microbial chemotherapy and the emergence of microbial resistance has re-focused attention to passive immunization as a way of controlling infection. For this purpose, the egg antibody has received much attention in recent years. From a practical standpoint, passive immunization using egg antibodies entails less cost than production of cow colostral or serum antibodies. Compared to chemotherapeutic agents, antibody usage is not expected to generate resistance by microorganisms and an unwanted antibiotic residue in meat is avoided. Egg antibody is well tolerated in pigs and the site of action is localized in the intestinal tract rather than systemic. Oral passive immunity with anti-fimbrial antibodies essentially prevents bacterial adherence by interfering with the surface localized fimbriae from adhering to intestinal receptors. The net effect is reduced extent and intensity of colonization precluding the overt expression of clinical disease. Recently, we have shown that the use of orally administered egg antibodies directed against the K88, K99, and 987P fimbriae of *E. coli* effectively prevented diarrhea in neonatal isolated pigs [12]. In this report, we extend this study by using egg antibodies directed against the F18 fimbriae involving weaned pigs.

MATERIALS AND METHODS

Bacteria and cultivation conditions: *E. coli* strain 8199 (O141ab: H4: F18ac$^+$: STIa, STII) [9] was obtained from Prof. Bertschinger, Institute of Veterinary Bacteriology, University of Zürich, Switzerland. The bacteria were grown on Iso-Sensitest Agar (Oxoid, Unipath Ltd., Hampshire, England) containing the dye Alizarin yellow R (Sigma, MO, U.S.A.), 0.0625% w/v for 20 hr at 37°C in an atmosphere containing 5% CO_2 [11]. The bacteria were suspended in phosphate-buffered saline (PBS; pH 7.2), the fimbriae detached by incubating the suspension in a water bath at 60°C for 20 min with shaking (50 rpm), and the bacterial cells removed by centrifugation for 20 min at 12,000 × g, 4°C. The fimbriae were precipitated by adding 20% v/v of saturated ammonium sulfate followed by centrifugation for 20 min at 12,000 × g. The pellet was resuspended in 0.1 M Tris-HCl buffer (pH 8.8) and dialyzed against the same buffer. The suspension was applied to a chromatographic column packed with QAE-Sephadex A-50 (Pharmacia, Uppsala, Sweden). The column was equilibrated with 0.1

M Tris-HCl buffer (pH 8.8). For fractionation, 0.1 M Tris-HCl buffer (pH 8.8) with increasing NaCl concentrations (0.3, 0.5, 0.7, and 1.0 M) was used for elution and were fractionated while monitoring protein concentration spectrophotometrically at 280 nm. The eluted fimbrial suspension was pooled and dialyzed against PBS and the protein content of the pooled sample was determined by a Bio-Rad protein assay system (Bio-Rad Laboratories, CA, U.S.A.). The purity of fimbrial preparation was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in 15% acrylamide gels with pre-stained standards (Bio-Rad). Purity of the fimbriae was further confirmed by transmission electron microscopic (Model H-300; Hitachi, Tokyo, Japan) examination of negatively stained samples. Protein-containing fractions were tested by immunoblot using anti-F18ac antibody obtained from Prof. Bertschinger.

Egg antibody production: A total of five-month-old White Leghorn chickens (strain HyLine W36; GHEN Corporation, Gifu, Japan) in conventional isolated poultry housing were immunized for egg antibody production. The fimbrial vaccine was prepared by mixing 0.5 mg of fimbrial antigen with 0.5 m*l* emulsion oil containing 5% Arlacel 80 (Maine Biological Laboratories, Waterville, ME, U.S.A.). The vaccine (1 m*l*/dose/bird) was injected intramuscularly with 0.5 m*l* injected to each of the breast muscle. Eight weeks after the initial injection, the booster was given in the same manner. The eggs were harvested daily starting 2 weeks after the booster and stocked at 4°C until yolk collection. The egg yolks were separated from the egg white and yolk membrane and pooled. The pooled egg yolk was homogenized with a mixer (HVM-106; Nihonseiki Kaisha, Tokyo, Japan) and filtered by Teflon filter cloth (Asamasu Co., Ltd., Nagoya, Japan). The filtrate was applied to a spray-dry machine (Model L-12; Ohkawara Kakohki, Kanagawa, Japan) which was operated at air-inlet temperature of 140°C. At the bottom of the dryer, the dried material was transported by a flow of air at 72°C to the collection vat. The dried antibody powder was stored in a desiccator at room temperature until use.

Titration of antibodies by enzyme-linked immunosorbent assay (ELISA): ELISA was performed as previously described [12]. Microdilution plates (Immulon 2; Dynatech Laboratories, Alexandria Va, U.S.A.) were coated with 100 μ*l* per well of a 5 μg/m*l* solution of purified fimbrial antigen in 0.05 M carbonate buffer (pH 9.6) at 4°C for 18 hr. The plates were emptied and blocked with 150 μ*l* per well of PBS containing 3% bovine serum albumin at 37°C for 1 hr and then washed with 0.02% Tween 20-saline three times. Sample antibody powders and test feed mash were reconstituted or suspended in PBS (1:100 dilution). For feed mash, the sample was centrifuged (100 × g, 15 min) and the supernate was collected for antibody titration. Twofold serial dilutions of this working antibody solution in 0.05% Tween 20-PBS were then dispensed to the blocked microplate wells (100 μ*l*/well) and incubated at 37°C for 1 hr and the plates were washed as described above. Rabbit anti-chicken IgG conjugated with horseradish peroxidase (Cappel, Organon Teknika Co., PA, U.S.A.) diluted 1:8,000 in 0.05% Tween-PBS was applied and incubated at 25°C for 30 min; Thereafter, the o-phenylenediamine and dihydrochloride was added. The color reaction was stopped after 20 min with 3N sulfuric acid, and the intensity of color developed was measured at 490 nm with a microplate reader (Model MR 5000; Dynatech). The titer was defined as the dilution of the antibody sample that yielded an A490 optical density (O.D.) of 0.2 in a two-fold serial dilution curve.

Challenge procedure and treatment with anti-F18 antibody: A total of 74 weaned, F18 antibody-free four-week-old Large White pigs derived from a total of 7 litters were obtained from a contractual pig grower of GHEN Corporation. Feeds were purchased from Standard Experimental Diets for Piglet SDS-No.1 (Nihon Haigou Siryou, Aichi, Japan). The pigs were kept in separate flatdeck holding pens in isolation rooms held at 20°C. They were randomly distributed into three groups: Group 1 (28 pigs) as non-treated control; Group 2 (18 pigs) given antibody with 1:10 titer pre-mixed with feed ration; Group 3 (28 pigs) given antibody with 1:50 titer in feed as in group 2. The pigs were challenged orally using a 20-m*l* syringe attached to the silicone tubing. Chall1enge was done once daily for three days with 10^{11} cfu of viable *E. coli* 8199 (F18ac$^+$ homologous strain) on day 0. Starting from challenge day until day 9 everyday, the treatment groups were given the antibody-supplemented feed as specified above. The clinical response of each pig was noted throughout the experiment in terms of presence and severity of diarrhea, weight loss, and enumeration of F18$^+$ *E. coli* from rectal swabs. The presence of diarrhea was based on fecal consistency score using the following index: 0, normal feces (i.e., firm and well formed); 1, soft feces (i.e., soft and formed consistencies); 2, mild diarrhea (i.e., fluid, usually yellowish feces); 3, severe diarrhea (i.e., watery and projectile feces). The degree of colonization of the intestine (duodenum, jejunum, ileum, cecum, and colon) by F18$^+$ *E. coli* in pigs sacrificed on day 9 was evaluated by culturing intestinal swabs taken at the time of necropsy of all test pigs.

Isolation and identification of F18-positive E. coli: Rectal swabs were taken at 0, 2, 4, 7, and 9 days after infection. The challenge F18$^+$ *E. coli* strain was isolated by culturing the swab specimens on desoxycholate-hydrogen sulfide-lactose agar (DHL agar; Eiken Chemical Co., Ltd., Tokyo, Japan) plates and Trypticase soy agar plates with 5% defibrinated sheep blood in an atmosphere containing 5% CO_2. The F18$^+$ *E. coli* strain was detected by slide agglutination test using an F18 fimbriae-specific antiserum. A pig was considered to be excreting F18$^+$ *E. coli* if this organism predominated in the culture.

In vitro adhesion assay: The succeeding procedure in the adhesion assay was performed as described elsewhere [12]. Isolated small intestine epithelial cells were prepared from three-week-old pigs. The intestinal contents were washed

with PBS, and filled with EDTA buffer solution (8 mM KH_2PO_4, 6.5 mM KCL, 10 mM EDTA {pH 6.8}) until fully distended. The distended intestine was then immersed in PBS and incubated at 37°C for 5 min with shaking. The intestinal segment was then longitudinally split open and the mucosal surface was gently scraped with a microscope slide several times to remove the remaining epithelial cells; each cell scraping was added to the previous collection. Epithelial cells were then washed once with Hanks balanced salt solution (HBSS), centrifuged at 100 × g for 10 min at 4°C and the resulting pellet was resuspended for cell counting. One m*l* of the epithelial cell suspension containing about 10^6 cells was added to 1 m*l* of 10^8 cfu of $F18^+$ *E. coli* suspension and incubated at 37°C for 30 min. Nonadherent bacteria were removed by four wash repetitions with centrifugation at 100 × g for 10 min in each wash. The final cell pellet was suspended in 4 m*l* of HBSS and 1 m*l* aliquots of the epithelial cell: bacteria mixture were dispensed onto a Lab-Tek chamber slide (Nunc Inc, Ill. U.S.A.). Cells in the chamber slide were centrifuged at the same speed as described above to allow adherence to glass well bottom, fixed in ice-cold ethanol for 15 min, stained with Giemsa for 30 min and then examined under light microscope. The number of bacteria attached to 25 epithelial cells was determined and the average number of bacterial cells attached to a single epithelial cell was calculated. Bacterial attachment to epithelial cells was inhibited with anti-F18 fimbrial antibody preparations by preincubating 1 m*l* of bacterial suspension with 1 m*l* of antibody solution (1:950 ELISA titer) for 15 min after which 1 m*l* of epithelial cells was added and then incubated at 37°C for 30 min.

Statistical analysis: The statistical significance of differences in fecal consistency score and bacterial count between the treated and control groups was assessed by Fischer exact test. Differences in weight gain and *in vitro* adherence to epithelial cells between the treated and control were assessed by Student's *t*-test.

RESULTS

The F18 fimbrial antigen obtained using the present method of purification was highly purified as revealed by SDS-PAGE and Western blot analysis showing minimal contamination with other bacterial protein (data not shown). Transmission electron microscope findings showed that negatively stained fimbriae were characteristic of fimbrial structures (data not shown). The chicken egg antibody powder produced by spray drying was tested for immunoreactivity by ELISA and gave a titer of 1:950 after reconstituting the powder by 1:100 dilution using PBS.

The body weight gain of challenged pigs subsequently treated with either one of two concentrations of antibodies (groups 2 and 3) in feed against the control (group 1) is shown in Table 1. Groups 1 and 2 had comparable weight gain at 52% while group 3 pigs had about 80% which was significantly higher than those of the controls ($P<0.01$). The difference in frequency of diarrhea (Table 2) between groups 1 and 3 was significant at 2, 3, and 4 days after challenge ($P<0.05$–0.01) while that of group 2 was significantly different from the control only at 2 days after challenge ($P<0.05$). By fecal scoring, group 3 pigs had less severe diarrhea ($P<0.01$) and shorter duration of diarrheic episode against control group (Table 3). Group 2 had almost the same frequency of diarrhea as the control pigs but the fecal score peaked 2 days later than the controls (day 4) to a level similar to that of the control on that day. Group 1 had the highest overall mean fecal score which peaked at 2 days

Table 1. Body weight gain after challenge exposure to $F18^+$ *E. coli* among pigs treated with different concentrations of antibody powder

Group	No. of pigs	Antibody titer post-mixing in feed[a)]	Body weight gain (%)[b)]
1	28	<1:1	52.4 ± 27.8
2	18	1:10	52.1 ± 19.1
3	28	1:50	79.9 ± 31.3**

**: $P< 0.01$ relative to Group 1 (Student's test).
a) Sample used was 1:100 dilution of antibody supplemented feed.
b) % Body weight gain ={(weight on day 9 – weight on day 0)/weight on day 0} × 100.

Table 2. Presence of diarrhea after challenge exposure with $F18^+$ *E. coli* among pigs given different concentrations of antibody powder in feed (Groups were the same as in preceding tables)

Group	No. of pigs	Percentage of pigs with diarrhea on clinical observation day									
		0	1	2	3	4	5	6	7	8	9
1	28	0	32	46	43	39	11	11	11	11	11
2	18	0	11	11*	22	28	17	17	11	11	6
3	28	0	21	4**	11*	7*	0	0	4	4	0

*: $P<0.05$, **: $P<0.01$ relative to Group 1 (Fischer exact test).

Table 3. Severity of diarrhea after challenge exposure with F18+ *E. coli* among pigs given different concentrations of antibody powder in feed (Groups were the same as in preceding tables)

Group	No. of pigs	Average of fecal score on clinical observation day[a)]									
		0	1	2	3	4	5	6	7	8	9
1	28	0	0.68	0.82	0.57	0.54	0.21	0.14	0.11	0.11	0.11
2	18	0	0.22	0.11	0.33	0.56	0.22	0.17	0.11	0.11	0.06
3	28	0	0.29	0.4	0.11	0.07	0	0	0.04	0.04	0

a) Calculated as Total daily group score/No. of pigs in the group.

Table 4. Frequency of isolation and quantitative recovery of F18+ *E. coli* from intestinal swabs of challenged pigs (Groups were the same as in preceding tables)

Group	No. of pigs	Percentage of rectal swabs positive on observation day (average $\log_{10}$ cfu/g positive stool swab)					Percentage of swabs positive on day 9 (average $\log_{10}$ cfu/g positive stool swab)				
		0	2	4	7	9	Duodenum	Jejunum	Ileum	Cecum	Colon
1	28	0	100 (8.9)	89 (7.8)	61 (6.5)	14 (6.3)	4 (3.0)	4 (4.3)	14 (4.8)	14 (5.3)	14 (6.8)
2	18	0	100 (8.4)	72 (8.0)	11** (5.6)	0 (<3.0)	0 (<3.0)	0 (<3.0)	0 (<3.0)	6 (4.1)	6 (4.2)
3	28	0	100 (8.8)	32** (7.0)	4** (4.7)	0 (<3.0)	0 (<3.0)	4 (3.0)	4 (3.0)	4 (3.3)	4 (3.0)

**: $P< 0.01$ relative to Group 1 (Fischer exact test).

Table 5. Effect of anti-F18 fimbrial antibody on *in-vitro* adhesion of F18+ *E. coli* to pig small intestinal epithelial cell

Bacterial suspension	Number of bacteria attached per epithelial cell	
Control (not pre-incubated with antibodies before assay)	15.3 + 8.2	(100%)
Pre-incubated with F18 antibody (titer 1:950)	4.5 + 2.1**	(29.4%)

**: $P<0.01$ relative to control (Student's test).

post-challenge then gradually waned and was still seen in 11% of pigs until the last day of observation.

However, although groups 2 and 3 pigs were generally protected from fatal infection. Some of them were still excreting the bacteria in the feces and harboring F18+ *E. coli* in the intestine at the end of the study based on isolation from rectal swab specimens and from intestines of sacrificed pigs. The frequency of isolation of F18+ *E. coli* from rectal swabs in group 3 was significantly low ($P<0.01$) at 4 and 7 days after challenge from those of the control group while group 2 was significantly different only at 7 days ($P<0.01$) (Table 4). Quantitative isolation of *E. coli* from rectal swabs during the clinical observation period and intestinal tissues post-mortem revealed an almost similar bacterial load among positive pigs in the control compared to antibody treated pigs (Table 4).

F18+ *E. coli* adhered to isolated pig small intestinal epithelial cells. It was observed to attach to the brush border as well as lateral and basal borders of epithelial cells. The average number of adherent bacteria per epithelial cell was 15.3 ± 8.2 (Table 5). The attachment of bacteria to epithelial cells was significantly reduced ($P<0.01$) by 70% using homologous anti-F18 fimbriae antibody from chicken egg.

DISCUSSION

The present data on the use of oral anti-F18 fimbrial antibody for immunotherapy extends the earlier observation on the therapeutic efficacy of anti-fimbrial antibodies (K88, K99, and 987P) against intestinal colonization in *E. coli* infected pigs [12]. In the present protocol, the effect of antibody was therapeutic in the sense that existing infection seen on days 4 to 9 was diminished upon continued antibody administration based on well define clinical parameters. On the other hand, the concurrent administration of antibody and *E. coli* on days 1 to 3 indicates that part of the efficacy may be preventive. The present data showed that pigs can be protected by oral antibody from loss of body condition following infection with the pathogenic strain of F18+ *E. coli*. Supplementation of feed with egg antibody specific

for F18 fimbriae concomitantly reduced the frequency, severity and duration of diarrhea as well as the frequency of excretion of infective *E. coli* in test pigs. These effects were all observed in a dose-dependent manner which confirm the specificity of antibody activity. Although there was similarity in bacterial load among positive test pigs in Group 3 compared to control based on isolation from rectal swabs and post-mortem intestinal tissues, the frequency of excretion of *E. coli* was much lower ($P<0.01$) among those treated with antibody than in control pigs on days 4 and 7 (Table 4). The magnitude of challenge used in this study did not result in mortality which may be related to the degree of pathogenicity of the strain used. In this experiment, we used a strain that was initially isolated from the field [9]. We did observe frank diarrhea with varying degrees of severity in about half of the susceptible pigs used in initial challenge testing (data not shown) and in the control group. It is possible that reduction of bacterial load as observed in this study may occur even when the challenge strain is more toxigenic than the strain we used here. This is due to the fact that protection by anti-fimbrial antibody is dependent on the expression of surface F18 fimbriae and targeting this fimbriae is expected to preclude colonization and therefore subsequent toxin production regardless of the degree of toxigenicity of the strain.

The use of orally administered crude yolk antibody for feed supplementation is a convenient and cost-effective way of treating infections in the field compared to parenteral antimicrobial agents. Extending the use of oral egg antibody to other diseases of the gastrointestinal tract of domestic animals have been tried in recent years [3, 6, 10] and may gain more importance in the future.

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