RESEARCH REPORT
SWINE HEALTH

Title: Environmental Factors that Induce the Expression of Receptors for F18+ Enterotoxigenic *Escherichia coli* - **NPB #03-090**

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Abstract: Some pigs are susceptible to F18+ the enterotoxigenic *Escherichia coli* (ETEC) that produce F18 fimbriae by genetic heritage, while others are inherently resistant to that organism. Those that inherit the gene for susceptibility become at risk to disease at weaning. The factor or factors that render these pigs at risk at weaning are only partly understood, but appear to involve expression of the F18 receptor to which the fimbriae attach in colonizing the piglet intestines. In the current study, we sought to identify the environmental cues responsible for expression or activation of the F18 receptor. We reasoned that these environmental cues might be furnished by bacteria of the intestinal flora or constituents of the weanling diet, or both. Either the change to the weanling diet results in a change in the types of bacteria that inhabit the pig's intestines and these bacteria affect disease susceptibility, or the high starch content of the weanling diet has a physiological effect on the pig intestines that results in increased susceptibility to infection and diarrhea. For this study, pigs were reared germ-free from birth for three weeks then given sterile weanling pig feed, a defined population of intestinal bacteria reflective of the intestinal flora, or both. One week later, pigs were challenge-inoculated with F18+ ETEC. None of the pigs developed diarrhea. However, when we measured the number of the pathogenic bacteria in the intestines of pigs receiving either the intestinal bacteria or the sterile weanling pig diet, we observed a significant increase. Further, when we measured the ability of F18+ *E. coli* to adhere to the brush border surface of intestinal epithelial cells from pigs given either a bacterial flora or the weanling diet, we observed an increased ability for the pathogenic bacteria to adhere. The observations of this study suggest that changes in diet and microbial flora at the time of weaning both contribute to susceptibility of pigs to enterotoxigenic *E. coli*. These and subsequent studies may lead to recommendations for the alteration of the weanling diet to reduce susceptibility to colibacillosis without compromising pig performance.

Introduction: The current research effort was to determine whether environmental factors including intestinal flora bacteria and/or the diet typically offered weaned pigs might contribute to the expression of receptors to which F18 fimbriae of enterotoxigenic *E. coli* (ETEC) binds in the intestines of young pigs. The fimbrial binding structure on porcine intestinal epithelial cells exploited by F18 fimbriae as a receptor includes the sugar fucose, the expression of which appears to be essential for bacterial attachment in the intestines. The enzyme fucosyltransferase-1 (FUT-1) is responsible for covalent linkage of the sugar to the rest of the receptor complex. However, the enzyme is apparently not expressed constitutively, but must be induced by events in the intestine. In gnotobiotic (germ-free) mice, bacteria including *Bacteroides thetaiomicron* that utilize fucose as a carbon source induce expression of FUT-1 (Bry et al, 1996). Thus, growth of fucose-utilizing bacteria in the intestines of mice results in the production of molecules on the epithelial surface of the intestines that contain fucose. Those molecules are known as glycoconjugates and are expected to be similar to the F18 receptors expressed by pigs. Other studies have shown that an increase in the production of insulin, associated with introduction of a high carbohydrate load in the gut (such as the weanling diet) induced an increase in glycosylation on gut epithelium (Lenoir et al., 2000). This glycosylation apparently includes the attachment of fucose sugars to proteins and lipids forming glycoconjugates associated with epithelial cell membranes in the intestines. These observations lead us to hypothesize that either a change in intestinal flora favoring fucose-utilizing bacteria, or the change in carbohydrate load coincident with weaning resulted in the expression of fucose-containing receptors for F18 fimbriae, thus increased susceptibility of piglets to F18+ enterotoxigenic *Escherichia coli*. To address this hypothesis we established two objectives:

Project Objectives:

1) To determine whether *Bacteroides thetaiomicron*, an intestinal bacterium that induces alpha (1,2) fucosyltransferase (FUT-1) expression in germ free mice can induce F18 receptor expression in pigs and render those animals susceptible to F18+ enterotoxigenic *Escherichia coli* (ETEC) challenge (FUT-1 induction appears to play a critical role in F18 receptor expression).
2) To determine whether the weanling diet alone, or in combination with a defined complex intestinal bacterial flora can induce F18 receptor expression and render pigs susceptible to F18+ ETEC challenge.

Materials and Methods: To test whether either *B. thetaiotaomicron* or an intestinal flora was capable of inducing the expression of F18 receptors in pigs, we inoculated gnotobiotic (germ-free) pigs with a cocktail of bacterial strains including *B. thetaiotaomicron,* plus *Enterococcus faecalis, Streptococcus bovis, Chlostridium clostridioforme*, *Lactobacillus brevis* and *Escherichia coli.* All strains were purchased from the American Type Culture Collection, except the *E. coli* (Strain G58-1, which we use in our laboratory as a non-pathogenic control) and *B. thetaiotaaomicron* (received from J.I. Gordon, Washington University, St. Louis, MO). Bacteria were propagated on appropriate media to approximately 1 x 10^9 colony forming units (CFU)/ ml and one ml/strain was given to pigs when they were 3 weeks old. The *B. thetaiotaomicron* strain uses L-fucose or D-arabinose as a carbon source and induces fucosyltransferase (FUT1) expression in germ-free mice Bry et al, 1996). Induction of the FUT1 gene is believed to be essential for expression of F18 receptors as explained above.

To test where the weanling diet alone, or in concert with a microbial flora, composed as described above could induce F18 receptor expression and render pigs susceptible to F18+ ETEC challenge, we provided 3-week-old gnotobiotic pigs with a sterile weanling diet, or a mixed microbial flora in addition to the sterile weanling diet. Other piglets were maintained on milk replacer germ-free. All pigs in both experiments were challenged with approximately 3 x 10^9 CFU of an F18+ ETEC strain (Strain 2134) seven days after flora-inoculation and/or placement of weanling diet.

Following challenge, animals were monitored for clinical signs of enteric disease, including diarrhea, dehydration, anorexia, and lethargy. Animals were euthanized 72 hrs after infection and subjected to necropsy and examined for evidence of disease, including fluid filled colons, and hyperemia or edema (reddening or swelling) of internal organs. Intestinal tissue specimens (duodenum, jejunum, ileum, and cecum) were collected for histological analysis and ileum was collected for quantitative culture of β-hemolytic *E. coli* (the F18+ ETEC strain 2134) and immunofluorescence for bacteria expressing F18 fimbriae (by the challenge strain 2134). A specimen of jejunum was also collected to test for the expression of F18 receptors, by means of the brush border adherence assay (Francis et al., 1998). An ear specimen was collected to test for the F18 receptor gene marker. Histological sections were examined for adherent bacteria and for histological lesions. To determine whether the pigs carried the genetic marker for the F18 receptor, F18 receptor genotyping was done by PIC International Group, Health Biotechnology Research, Berkeley, CA. using the ear specimen collected from each pig at euthanasia.

Piglets for this study were delivered by closed hysterotomy, maintained germ-free in sterile rigid tub isolators, and fed a sterile commercial neonatal animal formula (ESBILAC-Lac, PetAg, Inc, Hampshire, IL). Rectal cultures of piglets are taken weekly to check for presence of contaminating bacteria. Piglets were observed at least 2 times daily for general health and well-being. Euthanasia was performed by a method approved by the American Veterinary Medical Association. Pigs weaned from milk replacer at 3 weeks were fed a sterile diet typical of that for conventional pigs their age. The diet met all their nutrient requirements (as per NRC, 1998) and was offered in a wet meal form (gruel) with no antimicrobial product added. Corn, lactose, and soybean oil were the main energy sources, and soybean meal (46.5% CP), dried whey, and porcine plasma were added for supplementation of amino acids. Sterilization was done by autoclaving. Filter-sterilized vitamins and minerals were added after the feed was autoclaved.

An effort was made to assign equal numbers of pigs to each treatment group, but this effort was complicated by the fact that only at the conclusion of the study after pigs were euthanized, were we able to determine the genotype of pigs with respect to FUT-1 marker the F18 receptor. Eight pigs were determined by these tests to be genetically incapable of producing the F18 receptor and these pigs were retrospectively pooled into one group regardless of the treatments (diet of flora) the pigs received. Twenty eight pigs that were genetically able to produce F18 receptor (that is they had an intact FUT-1 gene). Number of animals in the various treatment groups is shown in table 1.

Table 1. Treatment groups for gnotobiotic piglets used in this study.

Treatment group	No. of Pigs
FUT-1 gene neg. (pigs may have received any treatment)	8
FUT-1 gene pos.	
Negative Control (not given flora and not weaned to solid diet)	6
Normal Flora (pigs given a mixture of bacteria, but not weaned to solid diet)	8
Weanling Diet (pigs not given mixture of bacteria, but placed on solid diet)	8
Normal Flora and Weanling Diet	6

Results: None of the pigs in the study developed clinically apparent diarrhea. Although some pigs had more fluid feces than did others, there were no treatment-specific differences in fluidity of the feces. However, there were significant differences between groups of pigs with regard to the concentration of F18 ETEC per gram of small intestine (ileum), and in the amount of F18+ bacteria detected by immunofluorescence in intestinal smears (Tables 2 & 3). First of all, pigs with a defective receptor gene (FUT-1 negative), were significantly less well colonized after challenge than were animals that possessed the gene, but received neither intestinal flora nor the weanling diet. Further we found that among piglets possessing the intact FUT-1 gene (FUT-1 positive), those receiving an intestinal flora (including *Bacteroides thetaiomicron*) became significantly more colonized with F18+ ETEC when challenge inoculated, than did pigs not given an intestinal flora. No such increase in colonization was noted among pigs receiving either sterile weanling pig feed, or both flora and weanling feed. No significant increases were noted in bacteria expressing F18 in intestinal smears taken from piglets after infection.

With regard to the expression of F18 receptors as detected through adherence of F18+ ETEC to brush border vesicles prepared from the intestinal epithelial cells of pigs in the study, significantly more bacteria adhered to brush borders isolated either from pigs receiving flora or feed than from pigs not possessing the functional FUT-1 gene (Table 4). Further, brush border vesicles from FUT-1+ pigs on weanling feed bound significantly more F18+ ETEC than did FUT-1+ pigs retained germ free and on milk replacer. Also, the greater number of bacteria adherent to brush border vesicles from FUT-1+ pigs given a normal flora approached significant difference from that bound to brush borders of the FUT-1+ pigs retained germ free and on milk replacer ($P=0.066$). Interesting, both colonization and receptor expression appeared to be suppressed when piglets were given both an intestinal flora and the weanling diet. This latter observation may suggest that some constituent in the intestinal flora suppressed the growth and activities of *Bacteroides thetaiomicron* when fermentable sugars were available in the diet.

Table 2. Means of the Colony-Forming Units (x 10^7) of F18+ ETEC per Gram of Ileum in FUT-1-Negative Pigs, and FUT-1-Positive Pigs Given an Intestinal Flora, Weaned to Solid Feed, or Both.

Pig genotype	FUT-1-Neg	FUT-1-Positive			
Treatment	All Treatments	None (Control)*	Flora	Weanling Feed	Flora and Weanling Feed
CFU/Gram Ileum	1.18 ±1.30	4.13 ± 4.85	44.7 ± 42.6†‡	5.43 ± 7.46	1.92 ± 4.35

*received milk replacer

†Significantly different from FUT-1 Neg P=0.023
‡Significantly different from Treatment-neg Control

Table 3. Means of the IFA scores (relative concentration of F18+ ETEC visualized by IFA) in the ilea of FUT-1-Negative Pigs, and FUT-1-Positive Pigs Given an Intestinal Flora, Weaned to Solid Feed, or Both.

Pig genotype	FUT-1-Neg	FUT-1-Positve			
Treatment	All	None (Control)*	Flora	Weanling Feed	Flora and Weanling Feed
Mean IFA Score	2.3 ± 1.0	2.1 ± 1.1	2.9 ± 0.32	2.7 ± 0.5†	1.2 ± 0.26

†Significantly less than the FUT-1positive; treatment-neg control

Table 4. Means of the Number of Bacteria Adherent on Ten Brush Border Vessicles Prepared from the Jejuna of FUT-1-Negative Pigs, and FUT-1-Positive Pigs Given an Intestinal Flora, Weaned to Solid Feed, or Both.

Pig genotype	FUT-1-Neg	FUT-1-Positve			
Treatment	All	None (Control)*	Flora	Weanling Feed	Flora and Weanling Feed
Mean No. Bacteria	1.3 ± 2.8	12.7 ± 12.1*	26.1 ± 12.0*	33.5 ± 6.2†	5.3 ± 7.2

*Significantly greater than the FUT-1-neg pig (P= 0.001; P<0.001, respectively)
†Significantly greater than the FUT-1positive; treatment-neg control (P=0.008

Discussion: The results of this study indicate that both diet and intestinal flora influence the expression of F18 receptors and inconsequence affect the extent of colonization of F18+ ETEC in the intestines of challenged pigs. However, these factors by themselves, at least in the form that was applied in this research cannot account for all of the changes that render some pigs exquisitely susceptible to F18+ ETEC at weaning. The number of bacteria adherent to brush borders from pigs most affected by presence of microbial flora or the weanling diet was still about an order of magnitude less than the number of K88+ ETEC we have observed on brush borders from pigs that are highly susceptible to K88+ETEC. Also, the number of F18+ ETEC adherent to the

isolated brush borders in the current studies is lower than the number of K8 ETEC observed adherent to the brush borders of enterocytes in consequence of clinical disease. In addition, we have identified two pigs (not of the current study) whose brush borders supported adhesion of F18 bacteria comparable to that observed with regard to K88+ETEC susceptibility. Further, the concentration of bacteria per gram of ileal tissue in the current study was about two orders of magnitude less than that observed in pigs severely affected with K88+ ETEC. Together, these observations suggest that greater adhesion of F18 ETEC must occur than was observed in this study before pigs will become clinically affected by the organism.

Based on the findings of the current study, and our observations made elsewhere that F18 ETEC adherence to the brush borders of pig enterocytes can be quite high, we hypothesize that expression of F18 receptors, thus susceptibility to F18 ETEC is multifactorial. We suspect that either a genetic factor in addition to the FUT-1 gene, or an environmental factor in addition to, or other than those we have assessed plays a central role in piglet susceptibility to F18 ETEC. Our data would suggest that a functional FUT-1 gene is essential to the production of the F18 receptor, and expression of the FUT-1 gene is driven by the bacterial flora and the weanling diet. In addition, another yet to be discovered factor appears to be required for full expression of the receptor. While that factor might be either genetic or environmental, we suspect it to be environmental based on the observation that F18 ETEC infection presents as a epidemic within a herd. That is, many pigs, perhaps most within some herds simultaneously become susceptible to infection. Currently, we are in search of pigs from herds with epidemic outbreaks and will examine these pigs for F18 expression. We will also compare environmental inputs that might make these pigs highly susceptible to F18 ETEC. We are also developing lectin histochemistry test to assess fucosylation the apical membrane of enterocytes in pigs receiving various treatments. Lectins specific for α-fucose may allow us to further differentiate changes in receptor expression between animals subjected to various environmental influences. Our studies will not be limited to F18 receptor expression analysis. It is possible that some other factor, in addition to the amount of F18 receptor expressed dictates pig susceptibility to F18 ETEC. One possibility is that the F18 receptor is partially or completely masked (covered up by some other molecule) in some pigs. Pigs develop resistance to ETEC disease regardless of the fimbria type (eg. 987P, K88, F18) with animal age and that resistance is unaccompanied by a reduction in receptor expression. A similar mechanism may be in operation with regard to susceptibility to F18 among weaned pigs.

References:

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Lay Interpretation:
This study was conducted to determine why pigs become highly susceptible to F18+ enterotoxigenic *E. coli* shortly after weaning. It was suspected that the change in susceptibility was triggered by the change in diet. Either the new diet results in a change in the types of bacteria that inhabit the pig's intestines and these bacteria affect disease susceptibility, or the high starch content of the weanling diet has a physiological effect on the pigs intestines that result in increased susceptibility to infection and diarrhea. Pigs were reared germ-free from birth for three weeks then given sterile weanling pig feed, a defined population of intestinal bacteria reflective of the intestinal flora, or both. One week later, pigs were challenge-inoculated with F18+ enterotoxigenic *E. coli*. Unfortunately, none of the pigs developed diarrhea. However, when we measured the number of the pathogenic bacteria in the intestines of pigs receiving either the intestinal bacteria or the sterile weanling pig diet, we observed a significant increase. Further, when we measured the ability of F18+ *E. coli* to adhere to the brush border surface of intestinal epithelial cells from pigs given either a bacterial flora or the weanling diet, we observed an increased ability for the pathogenic bacteria to adhere. The observations of this study suggest that changes in diet and microbial flora at the time of weaning both contribute to susceptibility of pigs to enterotoxigenic *E. coli*. We plan further work to more accurately determine what triggers susceptibility to colibacillosis at weaning. These studies may lead to recommendations for the alteration of the weanling diet to reduce susceptibility to colibacillosis without compromising pig performance.