RESEARCH REPORT
SWINE HEALTH

Title: Development of a Vaccine for F18+ Enterotoxigenic ***E. coli*** in Weaned Pigs – **r: NPB #04-026**

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Abstract In this investigation we attempted to establish that a topical (transcutaneous) vaccine delivery strategy pioneered in mice could be applied to intestinal tract infections of pigs, such as enterotoxigenic *E. coli*. Initial attempts to protect pigs from *E. coli* diarrhea by transcutaneous delivery of fimbrial antigen largely failed and the level an immune response to the fimbrial antigen was disappointing. Subsequent investigations were conducted in an attempt to optimize the method of topical vaccine delivery. Thus far we have not been successful in obtaining a desirable systemic or mucosal immune response, but are continuing to explore methods to optimize this method of vaccine delivery. Alternative methods to the delivery of vaccines to address diseases of the intestinal, respiratory, or urogenital tract (diseases of mucosal epithelium) all have limitations. Consequently, vaccines to such diseases are largely unavailable. Results from studies by other investigators using a mouse model suggest that topical vaccine delivery has substantial potential. However, our discussions with the developers of the mouse model suggest that perfecting the vaccine delivery method will be no trivial task. We plan continued work in this area.

Introduction: Bacterins and subunit vaccines for the protection of neonatal piglets from enterotoxigenic *E. coli* (ETEC) were developed nearly twenty years ago and have been so efficacious that the condition has nearly ceased to be an economic problem in the swine industry. However, the vaccine strategy used afforded protection to the young pig through immunization of the dam, who protected her offspring passively with antibodies in her colostrum. The piglets themselves remained immunologically naïve. This situation has led to the rise of a highly susceptible weaned pig population, who when commingled in nurseries, frequently fall victim to post-weaning colibacillosis. In consequence, this disease condition has become a major economic problem for many swine producers and occupies considerable management attention. The vast majority of the ETEC found in association with post-weaning diarrhea fall into either of two groups of bacteria classified by the fimbriae and enterotoxin virulence determinants that they express. These include K88 (fimbriae), and LT, STb ± STa (enterotoxins) strains found in about 50% of post-weaning colibacillosis cases and F18 (fimbriae), and STa, STb ± Stx2e (enterotoxins) found in about 40% of cases. The purpose of this research was to begin to explore the efficacy of a newly identified strategy in stimulating gastrointestinal immunity, thus protecting nursery pigs from the effects of infection with ETEC.

At the time this project was written, there were no commercially available vaccines from the protection of weaned pigs from post-weaning diarrhea. Since that time a live K88+ E. coli vaccine has been introduced. Its efficacy has not been independently verified, but may be similar to unlicensed strains with similar characteristics. Those strains have yielded mixed test results.

It is our objective in this current line of investigation to develop a vaccine that would meet the current needs of swine producers, achieve higher safety standards than the independently produced vaccines used today, and overcome the proprietary concerns of potential manufactures, thus inducing the industry to develop and license a commercial product. In this study, we tested several strategies for stimulating mucosal (intestinal) immunity, including a novel transcutaneous approach not yet attempted in swine, but showing promise in mouse models. Our approach was based on observations that enterotoxins (cholera toxin or *E. coli* heat labile enterotoxin-LT) are powerful mucosal adjuvants, and that when applied to decornified skin in a wet patch along with antigen, stimulate both circulating and mucosal antibodies. Further, IgA antibodies are delivered to the gastrointestinal tract in sufficient concentration that they exhibit a protective effect following experimental challenge with enterotoxin. Our long term goal is to develop such a vaccine that will stimulate protective antibodies against F18 and K88 fimbriae, and LT, STa and STb enterotoxins. The current investigation was an initial effort to establish efficacy of the concept for young pigs.

There is substantial literature indicating that either LT, the b subunit of LT, or the closely related Cholera toxin could serve as an effective mucosal adjuvant, enhancing immune responses to co-administered antigens. Particularly noteworthy are examples showing that this adjuvant effect may be obtained when the enterotoxin adjuvant (which itself is immunogenic) is delivered by application topically to the skin. Glenn et al. (1998) demonstrated that topical immunization (called transcutaneous immunization in the literature) with Cholera toxin protected mice against lethal oral toxin challenge. Antitoxin IgG and IgA antibodies were found in sera, lung washes, and more importantly, stool samples from immunized animals. The same group subsequently demonstrated the production of IgG and IgA antibodies to the colonization factor CS6 (a human ETEC equivalent of F18 or K88) in sera and stools of mice transcutaneously immunized with CS6 administered concurrently with Cholera toxin or LT. The antibodies recognized CS6 antigen, and the antitoxin (anti-LT) immunity induced by immunization protected against oral challenge with the enterotoxin (Yu et al, 2002). Both the antibodies to the adhesin and the enterotoxin would be anticipated to provide protection if administered to a susceptible host challenged with a live pathogen. However, lack of an adequate challenge model for human ETEC disease has hampered testing of the transcutaneous immunization concept for human pathogens. By contrast, availability of well defined models for ETEC disease in pigs made the current study attractive for testing the transcutaneous immunization concept for animal disease.

The mechanism by which transcutaneous immunization stimulates mucosal (intestinal) immunity is incompletely understood. However, apparently topically administered LT or Cholera toxin strongly stimulates the activities of Langerhans cells in the epidermis, which act as highly effective antigen-presenting cells (Guebre-Xabier et al., 2003). The topical LT or Cholera toxin, as an adjuvant induces migration of the activated antigen-presenting cells from the skin to the proximal draining lymph node, and antigen-presenting cells carrying the co-inoculated antigen either from the skin or from an injection site in the same anatomical region, also migrate to the same draining lymph node. Down-stream immunological activities in the lymph node result in the production of IgG and IgA antibodies against the inoculated antigens. IgA-producing lymphocytes migrate to lymphoidal tissues associated with mucosal surfaces including the intestines, and there participate the secretion of antibodies that have the potential of providing intestinal immunity.

Transcutaneous immunization offers several advantages over the use of a modified live vaccine. These include vaccine safety and enhanced protection for the manufacturer from piracy. Topical application of LT or Cholera toxin has proven safe in animal and human trials, and use of a transcutaneous subunit (specific antigen-based) vaccine precludes needle site abscesses and the possibility of a modified live vaccine strain acquiring virulence genes and returning to virulence. The antigen employed in the current study was purified ETEC fimbriae (K88). Purified and commercially available Cholera toxin was employed as the adjuvant. Future studies beyond the scope of this grant will explore efficacy of fusion proteins LT/STb and LT/STa and perhaps K88/LT and F18/LT in transcutaneous vaccines. Together these antigens are expected to protect against most postweaning ETEC strains.

Project Objectives:

1) To test the immune response of pigs to an ETEC F18 fimbria vaccine designed for transcutaneous delivery.

2) To test the ability of a transcutaneously delivered anti-F18 vaccine to protect pigs from infection by an $F18^+$ ETEC

As explained in our interim report, this project was modified to focus immunization on K88 in the place of F18 ETEC. The reason for the change was because experimental challenge with F18 ETEC has proven to be quite unpredictable. Challenge outcomes with K88 ETEC are more easily controlled, thus this organism is a superior model to test the currently proposed strategy of immunization.

Materials and Methods:

Procedures:

Fimbrial antigen-K88 fimbrial antigen was purified as described by Erickson et al (1992).

Bacterial Strains-Wild-type ETEC strain 3030-2 (K88/LT/STb; Francis and Willgohs, 1991) was utilized as a model challenge strain. The strain is highly virulent in pigs expressing the appropriate adhesion receptor from the neonatal period through at least 28 days of age. Assessment of pigs for the appropriate K88 adhesin receptor was done at necropsy using the brush border adhesion assay (Erickson et al, 1992). To ensure that a high proportion of piglets in the test litter had the receptor prior to enrollment in the project, intestines collected from the sow at euthanasia following C-section surgery were tested by the same assay.

Experimental animals- To preclude the influence of maternal antibodies and inadvertent inoculation of pigs with ETEC strains, the vaccine efficacy study was first conducted using Caesarian-derived, clostrum deprived piglets raised under gnotobiotic conditions. Inadvertent contamination of pigs with ETEC from the dam or other herd-mates has been a serious impediment to the use of conventionally reared pigs for this type of study (Francis and Willgohs, 1991). The sow was brought to our facilities at full term on the day of surgery. Piglets were delivered germ-free by closed hysterotomy, and maintained in sterile rigid tub isolators as previously described (Miniats and Jol. 1978). The animals were fed a sterile gnotobiotic piglet formula called ESBILAC-Lac (PetAg, Inc, Hampshire, IL). After 3 days, piglets were "conventionalized" with regard to bacteria representative of a normal intestinal flora and consisting of the following organisms: *Bacteroides thetaiotaaomicron, Enterococcus faecalis, Streptococcus bovis, Chlostridium clostridioforme*, *Lactobacillus brevis* and *Escherichia coli.* The *B. thetaiotaaomicron* was a gift of J.I. Gordon, Washington University, St Louis. The *E. coli* strain is a non-pathogenic wild-type control that we have used for many years (Francis, et al., 1986). The other strains were obtained from the American Type Culture collection. None of the bacteria are pathogens and collectively, they fail to cause any adverse reaction, including diarrhea, in 3 week-old gnotobiotic pigs (Francis, unpublished observations). The bacteria are used to help stimulate normal immune development (Christopher-Hennings, 1993; Butler 2000; 2002).

Further assessment of the immunization technology was conducted in conventional pigs brought to our facilities at weaning. Pigs were individually housed in stainless steel cages and fed weaned pig starter rations and provided water ad libitum.

Animal vaccination- Several methods were assessed for their ability to stimulate a systemic and mucosal immune response to E. coli antigens. The methods included 1) topical application of mucosal adjuvant and immunogens (transcutaneous immunization) 2) topical application of mucosal adjuvant over the site of subcutaneous injection of the antigen, and 3) interrectal inoculation of both mucosal adjuvant and antigen. The antigen was K88 fimbriae and the adjuvant was Cholera toxin. Topical application was done in a wet patch applied to skin either on the nape of the neck or midline abdomen. Bovine serum albumen (BSA) was used as a vaccine control antigen in some studies. Initial immunization procedures paralleled those used by other investigators in immunizing mice transcutaneously with *E. coli* colonization antigen CS6, influenza virus or heat labile enterotoxin (LT; Yu et al, 2002; Guebre-Xabier et al, 2003; Scharton-Kersten et al, 2000). Hair at the immunization site was shorn and the exposed skin subjected to mild abrasion with sand paper to disrupt the stratum corneum, then washed with water followed by 70% ethanol. A wet gauze patch containing 100 to 500µg Cholera toxin (Sigma

Biochemicals, St Louis, IL) and 300 µg fimbrial antigen in 200 µl PBS was taped to the prepared area and maintained overnight. The following day, the patch was removed and the skin washed to remove unabsorbed antigen and Cholera toxin. Animals were housed separately to preclude removal of patches by pen mates. Animals were vaccinated between 10 days and 20 days of age, with a booster immunization applied identically to the primary inoculation 10 days later. Pigs to be assessed for antibody production were euthanized 10 days after the second vaccination. Pigs to be assessed for protection from the effects of infectious disease were challenged orally with wild-type ETEC strain 3030-2 at the same time post-immunization. All challenge animals were tested for expression of the K88 receptors as described below.

Post-vaccination assessment- The efficacy of vaccine delivery methods were evaluated by measuring the specific immune response to the vaccine antigen, K88 fimbriae, and through protection afforded by immunization against challenge with $K88^+$ ETEC. Presence and concentration of IgM, IgG and IgA antibodies to K88 in serum and cecal contents of vaccinated pigs was tested for by ELISA. Monoclonal antibodies to K88 fimbriae were produced by us, isotype-specific anibodies against porcine immunoglobulins (IgM, IgG and IgA heavy chain) were obtained from Serotec, Oxford, UK.

Challenge inoculation of pigs- At four weeks of age, vaccinated pigs in a test litter were orally inoculated with approximately 3×10^9 colony forming units (CFUs) of $K88^+$ ETEC strain 3030-2. Prior to inoculation, blood was drawn from each pig to determine hematocryt and serum protein level. Differences in pre- and post challenge hematocryt and serum protein were used as correlates of the dehydration associated with severe diarrhea. Following inoculation, pigs were monitored for clinical signs of disease, including diarrhea, anorexia, visible dehydration and lethargy. Any pig becoming sufficiently ill that it failed to eat or stand was euthanized. All remaining pigs were euthanized at 96 hrs post inoculation. Blood was collected from all euthanized pigs for assessment of hematocryt, serum protein level, and antibodies. In addition, specimens of terminal ileum and mid jejunum were collect for histologic analysis (to assess bacterial colonization) and for epithelial cells from which to conduct K88 receptor analysis. Fluid cecal contents were also collected to assess antibody concentration.

Pigs were assessed for the expression of receptors that would make them susceptible to K88+ETEC. The test employed was the brush border adherence assay described by Erickson et al, 1992. That procedure assessed whether K88+ ETEC bacteria can adhere to the brush borders from epithelial cells removed from the small intestines of the piglet in question. Susceptibility is correlated with bacterial adherence.

Results: A litter of seven gnotobiotic pigs were reconstituted with an intestinal flora as described in the materials and methods section of this test, and then five of the seven pigs were vaccinated by the transcutaneous method also described above. The remaining two pigs were mock vaccinated and served as controls. The five vaccinates received 100 µg Cholera toxin (Sigma Biochemicals, St Louis, IL) and 300 µg K88 purified fimbral antigen in 200 µl PBS in an absorbent bandage patch taped to a prepared area on the nape of the neck and maintained overnight. Pigs received a second immunization by the same protocol 10 days later. Controls were mock vaccinated using PBS only. Pigs were challenged with a virulent ETEC strain 10 days following the second vaccination. Following challenge, piglets were monitored for dehydration by assessing change in hematocryt and total serum protein. Piglets were also observed for clinical signs of disease. Retrospectively pigs were assessed for presence of receptors to K88 fimbria. Only three pigs among the seven in the litter were found to possess K88 receptors, thus inherently susceptible to K88 ETEC. One of two susceptible pigs found among the vaccinates became diarrheic and the one susceptible control became diarrheic and died prior to scheduled euthanasia. Results of hematocryt test suggested that the sick pigs developed dehydration following challenge as shown in figure 1 below. Serum and cecum fluid immune responses are shown in table 1.

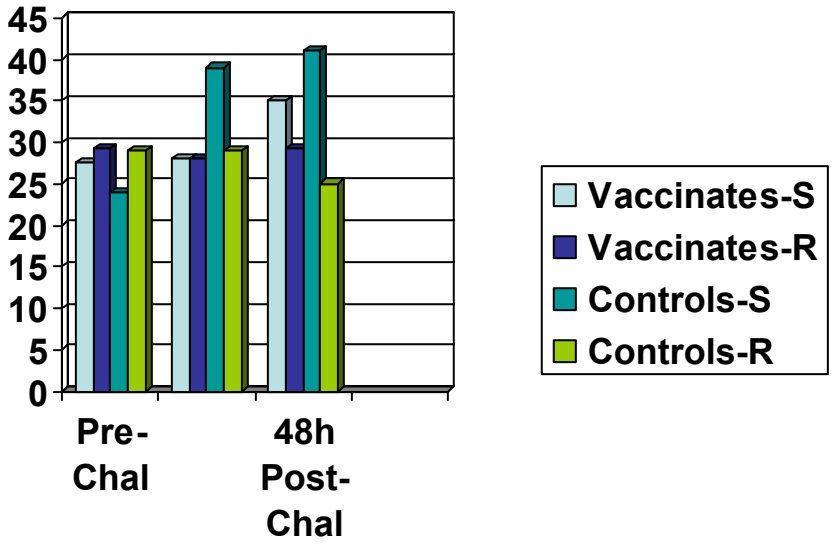

Figure 1. Pre-challenge and 24 hr and 48 hr post challenge hematocryts of vaccinated and non-vaccinated pigs were grouped by presence or absence of K88 receptors, thus the assumption of innate resistance (R) or susceptibility (S) to K88+ETEC. Hematocryt is given as percentage of blood volume. Susceptible pigs tended to exhibit a higher hematocryt following challenge suggesting dehydration. One of two vaccinated pigs failed to become ill, causing a trend for a lower hematocryt among those pigs, when compared to the only susceptible non-vaccinated pig.

Table 1. Immunoglobulin isotype-specific Anti-K88 antibody responses of vaccinated pigs as determined by ELISA test

	Vaccinates		**Non-Vaccinates**		
IgM	MeanOD	SD	MeanOD	SD	P value
pre-vax	0.411	0.22	1.022	0.49	
pre-2nd vax	3.2178	1.60	1.779	1.36	
pre-challenge	3.8094	0.43	2.4815	0.92	0.0370
Necropsy	2.8304	1.71	2.2365	0.16	0.6624
cecal fluid	0.4894	0.26	0.321	0.090	0.4310
IgG					
pre-vax	0.4338	0.20	0.538	0.18	
pre-2nd vax	1.1916	0.47	1.1395	0.33	
pre-challenge	2.9708	0.66	3.6835	0.27	0.2178
Necropsy	3.0558	1.65	4.09	0.59	0.4470

cecal fluid	0.4274	0.33	0.3605	0.16	0.8006
IgA					
pre-vax	0.2676	0.077	0.1995	0.023	
pre-2nd vax	0.5802	0.13	0.5395	0.090	
pre-challenge	1.2628	0.40	1.0615	0.56	0.6061
Necropsy	1.5596	0.60	1.523	0.83	0.9490
cecal fluid	0.6816	0.43	0.6235	0.41	0.8771

Increase in ELISA value between the time of the first vaccination and necropsy after completion of all vaccinations would suggest an increase in antibody titer, although titration is yet to be completed. ELISA optical density values above 1.5 may be beyond the linear scale of the test and are probably not a true reflection of antibody titer. Never-the-less, the most important observations are the values for antibody concentrations in the cecal fluid as they reflect immune protection in the compartment where the disease occurs. The results for cecal antibody shown here suggest little if any production in response to immunization. Differences between vaccinates and non-vaccinates with regard to all anti-K88 antibody isotypes were not significant.

Having observed minimal mucosal (or systemic) immune responses in consequence of vaccination by the transcutaneous method as described in the methods section of this document, we recognized a need to pursue optimization of the vaccine method and to examine other possible methods of vaccine delivery. To begin to accomplish this, we conducted two additional vaccine trials, in each case testing different parameters of vaccine delivery. Each experiment enrolled 12 newly weaned pigs (of about 20 days of life). For the first of these tests, some of the pigs were again vaccinated by the transcutaneous method. Others received antigen and adjuvant delivered by interrectal inoculation. The rectal area is rich in lymphoidal tissue, and interrectal inoculation has been demonstrated by other investigators to elicit a mucosal immune response. For our study, pigs were fasted for about 12 hrs, then inoculated by inserting a pipette into the rectal cavity and delivering the antigen (purified K88 fimbriae) and adjuvant (Cholera toxin). Antigen and adjuvant concentrations were the same as used for transcutaneous delivery. The same vaccination and sample collection time table was employed in the present study as was used in the first vaccine study, only piglets were not challenged with a virulent ETEC before experiment termination. Only blood and cecal fluid were collected for antibody assessment. Serum and cecum fluid immune responses as determined by ELISA assays are shown in table 2.

IgM

IgM	Rectal	SD	Patch	SD	Control	SD
pre-vax	0.3985	0.40	0.3493	0.47	0.4676	0.53
Necropsy	1.5719	1.80	1.7023	1.83	1.7719	1.70
cecal fluid	0	0.028	0	0.089	0.0055	0.12

IgG

IgG	Rectal	SD	Patch	SD	Control	SD
pre-vax	0.5673	0.43	0.783	0.34	1.4993	0.31
necrospy	1.8863	0.16	1.90	0.17	1.8483	0.12
cecal fluid	0	0.019	0	0.025	0	0.088

IgA

IgA	Rectal	SD	Patch	SD	Control	SD
pre-vax	0	0.091	0	0.039	0.0758	0.061
necrospy	0.4539	0.10	0.3263	0.13	0.2203	0.032
cecal fluid	0.0397	0.025	0	0.019	0.0375	0.080

P values for serum antibody analysis. Circulating anti-K88 antibody of the IgA isotype from pigs given rectal immunizations was significantly higher than from control pigs.

	IgA	IgG	IgM	.
Rectal vs. Patch	0.1051	0.7465	0.2367	
Rectal vs. Control	**0.0275**	0.7738	0.1297	
Patch vs. Control	0.3447	0.6180	0.5095	

Patch=transcutaneous immunization treatment group; rectal=interrectal immunization treatment group.

In this experiment, there appeared to be a serum immune response regardless of the immunization method used, although the response was quite weak. With a substantial response, one would have expected to have to dilute (serially) the serum in order to obtain an ELISA OD value in the readable range, which we did not. Thus, change in antibody titer was probably only marginal. No mucosal immune response was detected. Based on these observations, we reasoned that either the antigen and/or the adjuvant concentration was too low to elicit an immune response, or the skin thickness on the neck was too great. To address these issues in the last 12 pigs under experimentation, we selected a new site for inoculation were skin thickness was substantially less (abdomen), and applied various amounts of adjuvant from 100µg to 500µg/pig. We also elected to inject the antigen through the skin at the sight were the patch was applied to insure increased absorption of antigen. Subcutaneous injection at the site of transcutaneous adjuvant application has been used by other investigators (in a mouse model). This experiment is still ongoing and animal termination and final fluid collection is scheduled for July 8. Antibody assessment will follow shortly thereafter.

Discussion: Transcutaneous immunization has been shown to stimulate mucosal antibodies in mice and human beings, although protection against an infectious disease of the mucosal surface (such as ETEC) remains to be reported. However, we are aware that an experimental human ETEC vaccine trial was scheduled to be done this summer. Results for this study have not yet been made public. Having recently visited with a private research group focused on transcutaneous immunization, it has become quite clear that optimization of transcutaneous immunization is no trivial task and may take considerable effort. The investigation described above would support that conclusion. In these initial studies, we observed limited if any protection from challenge and minimal antibody production in response to transcutaneous immunization. Despite initial disappointing results, the effort to make this form of vaccine delivery work is probably worth the expenditure of time and resources, as there are few currently available alternatives and each comes with challenges and limitations. Alternatives to transcutaneous delivery that have or could be explored include live organism, or subunit oral delivery, and respiratory or rectal delivery. Live oral delivery of vaccines has the greatest promise of providing a robust and protective immune response. However, that method carries the greatest liability and economic risk. The live organisms with the greatest propensity for eliciting a robust and protective immune response are those that colonize or infect aggressively, hence are virulent. Attenuation reduces the strain's effectiveness. Highly attenuated stains, such as those that express K88 fimbriae, but lack enterotoxin genes have limited ability to protect against subsequent challenge (Francis and Willgohs, 1991). Spontaneous reversion to virulence carries a substantial risk, and the ease of unauthorized end user propagation of the vaccine strain dampens industry enthusiasm for developing and marketing live bacteria vaccines. Oral subunit vaccine development has been the subject of intensive investigation and appears to have limited practical

application due to a requirement for substantial dosage size and a tendency towards oral tolerance. Respiratory and rectal inoculation routes offer some promise, but the nature of delivery may makes obtaining consistent immune responses difficult to obtain. Construction and delivery of fusion proteins that combine the desired immunogenic antigen and a peptide that specifically targets a receptor plentiful on respiratory or rectal epithelium may be critical to a successful and consistent mucosal immune response. Hurdles to successful development of a transcutaneous-delivered vaccine may also be substantial, but data from mouse studies suggest the potential for efficacy. Hurdles to development of a transcutaneously delivered swine vaccine might include selection of a delivery site with skin more analogous to mice or humans (thinner and less cornified); determination of an optimal antigen and adjuvant dosage; engineering of a delivery vehicle requiring minimal (if any) site preparation, and identification of a less expensive adjuvant. With the study currently in progress, we are examining alternate application sites, attempting to optimize dosages, and improved delivery methods. Results from this study may provide directions of future work in this area, which will be followed up with funding that has been awarded from a different source. Once the optimum mucosal vaccine delivery system has been identified and proven effective, and we have had success regarding identification of an optimal mucosal vaccine delivery system, there will be substantial opportunities for application of the vaccination method in swine disease, as many of the infections of economic concern are infections of the mucosal surfaces. A partial list of potential disease targets includes enterotoxigenic E. coli, salmonella, rotavirus, TGE, PRRS, mycoplasma and Haemophilus.

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Lay Interpretation: In this investigation we attempted to establish that a topical vaccine delivery strategy pioneered in mice could be applied to intestinal tract infections of pigs, such as enterotoxigenic *E. coli*. Initial attempts to protect pigs from *E. coli* diarrhea largely failed and the level of immune response was disappointing. Subsequent investigations were conducted in an attempt to optimize the method of topical vaccine delivery. Thus far we have not been successful in obtaining a desirable immune response, but are continuing to explore methods to optimize this type of vaccine delivery. Alternative methods to the delivery of vaccines to address diseases of the intestinal, respiratory, or urogenital tract (diseases of mucosal epithelium) all have limitations. Consequently, vaccines to such diseases are largely unavailable. Results from studies by other investigators using a mouse model suggest that topical vaccine delivery has substantial potential. However, our discussions with the developers of the mouse model suggest that perfecting the vaccine delivery method will be no trivial task. We plan continued work in this area.