RESEARCH REPORT

PORK SAFETY

Title: Diagnosis and Prophylaxis of Clostridial Enteritides in Piglets **NPB# 06-136"**

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Industry Summary:
In a study entitled "The economic cost of major health challenges in large US swine production systems" (Holtkamp D et. al. AASV proceedings, 2007), *Clostridium perfringens* type A was ranked 3rd and *Clostridium difficile* 12th in terms of the most important health challenges in the breeding herd. Two previous studies in our laboratory had demonstrated that in piglets less than a week of age submitted to the Iowa State University Veterinary Diagnostic Laboratory with the complaint of diarrhea, *Clostridium difficile* and *Clostridium perfringens* type A were detected in 29-50% and 47-48% of the cases respectively and represent the two most commonly identified agents. For diseases believed to be of considerable importance, information on the pathogenesis, a reliable experimental model, and a gold standard for diagnostics are all lacking.
     The objectives of this study were to:
1. To further characterize the effect of *C. difficile* infection on the colon of affected piglets
2. To refine the diagnostics for *Clostridium perfringens* type A enteritis in swine.
3. To evaluate competitive exclusion as a means for preventing *Clostridium difficile* associated disease (CDAD)
4. To evaluate an experimental human CDAD vaccine, on which a pig product can be based

Clostridium difficile
     To critically evaluate the preferred sampling strategy for *C. difficile* diagnostics, 12 piglets, less than a week of age submitted to the diagnostic laboratory with suspect *Clostridium difficile*-associated disease (CDAD) were examined. Samples were collected from stomach, jejunum, ileum, cecum, 9 segments of colon, and rectum from each pig and submitted for *C. difficile* culture, C. difficile toxin ELISA, and microscopic examination. Data was analyzed to determine segments in which the correlation of these factors was the strongest.
     Clostridium difficile was isolated from stomach, small intestine and colon. A significant difference was not identified in the rate at which C. difficile was isolated from various segments of the gastrointestinal tract. Neither *C. difficile* toxin nor lesions were identified in stomach or small intestine. There was a statistically significant association between *C. difficile* isolation, detection of *C. difficile* toxins, and lesions in both the cecum and colon (p < 0.05), but not in the small intestine or stomach. Statistically significant differences between large intestinal sites in regards to *C. difficile* isolation, lesions, or toxin ELISA results were not identified.

Study results support the following conclusions. Cecum and colon remain the preferred sites for *C. difficile* diagnostics. A consistently superior sampling location in these sections of large bowel was not identified. All methods (toxin, culture, microscopic lesions) were relatively similar and typically detected 66-77% of affected pigs. Sampling of more than one site from each pig improved the diagnostic sensitivity to approximately 97%.

The use of fecal swabs as a tool for antemortem testing for *C. difficile* was evaluated. If sufficiently sensitive, this method would be preferred because it would eliminate the need to sacrifice piglets. Toxin was detected in 78% of fecal swabs from animals positive for *C. difficile* toxin in colon contents. This level of sensitivity is not adequate for individual animal diagnostics, but testing of fecal swabs for *C. diff* toxin may be useful way to diagnose *C. diff* issues on a herd basis.

Results of this study indicate that cases of *C. difficile* infection can be missed if only one sample from the large intestine is evaluated. If two or more samples are evaluated, the sensitivity appears to be quite high (97%). A preferred sampling location in the large intestine was not identified.

Clostridium perfringens type A:

Genotyping of *Clostridium perfringens* type A isolates from young pigs with diarrhea indicate that this disease appears to be due to strains of Clostridium perfringens that are positive for the beta2 toxin gene. Due to frequent lack of microscopic lesions, the occurrence of *C. perf* type A as normal flora, and the isolation of beta2-toxin positive strains from normal animals, the current diagnostic criteria for this disease are viewed by many as equivocal. A major aim of this study was to critically assess *C. perf* type A diagnostics and evaluate assays for beta2 toxin as a potential tool for the diagnosis of this disease.

For the purposes of this study, a pig was considered to have *C. perfringens* type A enteritis if the following criteria were met: 1) gross evidence for diarrhea, 2) exclusion of other infectious causes of diarrhea (*E. coli*, rotavirus, TGE, *C. difficile*, Salmonella, *C. perfringens* type C, PRRSV), and 3) a heavy growth of *C. perfrignes* type A was isolated from mid small intestine.

The following samples were collected from 10 pigs fulfilling these criteria and from 10 apparently healthy control pigs. Swabs were collected from the following sites for semi-quantitative *C. perfringens* culture: stomach, duodenum, small intestine at 10 cm intervals, ileum, and rectum. Contents were collected from each segment and fixed tissue was processes for microscopic examination. Ten *C. perfringens* isolates from each segment of bowel were analyzed by pulse filed gel electrophoresis. Fluid from each segment of bowel was analyzed for beta2 toxin using a dot blot analysis. In those pigs in which a heavy growth of *C. perfringens* was isolated from mid small intestine, the typical pattern of culture and microscopic lesions were as follows.

The level of bacterial growth was significantly higher in suspect *C. perf* type A pigs when compared to controls (2.39 ± 0.4 vs 0.64 ± 0.31; $p < 0.01$). Average percent of isolates producing beta2 toxin was higher in principals than in controls (87.2% vs 53.8%). Principal pigs had higher average lesion scores throughout small intestine (average score 0.52 ± 0.22) than controls (average score 0), but variation in lesion scores from proximal to distal small intestine was not significant. Gram-positive rods were noted in both principal and control pigs, although the average score was much higher in principals (1.12 vs 0.33). Beta2 toxin was detected by dot blot analysis in 23/300 (7.7%) of suspect *C. perf* type A pig samples and 19/300 (6.3%) of control pig samples.

We have proposed the following pathogenesis for *Clostridium perfringens* type A enteritis, based on these and previous results. The stomach acts as a fermentation vessel for the proliferation of *C. perfringens* with continuous seeding of the intestine resulting in high levels of *C. perf* throughout the entire small bowel. The lack of consistent villus atrophy or necrosis would suggest that released beta2 toxin results in a secretory diarrhea.

The results of this study provide support for parts of this proposed pathogenesis. The concept that *C. perfringens* type A enteritis is due to quantitative and qualitative changes

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in the intestinal population of Clostridium perfringens in supported by the higher numbers of *C. perf* type A harvested throughout the intestinal tract of suspect *Clostridium perfringens* type A pigs and the higher percentage of organisms positive for the beta2 toxin gene. The isolation of significantly higher numbers of beta2 toxin gene positive isolates from the stomach supports the concept that these quantitative and qualitative differences begin in the stomach.

Diagnostics for *C. perf* type A remain a central issue. Based on genotyping results, which have associate *C. perfringens* type A enteritis with beta2-toxin gene positive strains of the organism, we proposed that a test measuring beta2 toxin levels in the intestine could represent a useful diagnostic tool. Unfortunately, a statistically significant increased rate of beta2 toxin detection was not identified in affected pigs compared to control pigs. It does not appear that analysis for beta2 toxin will prove to be a useful test for the diagnosis of *C. perf* type A enteritis.

For ubiquitous organisms, such as *Clostridium perfringens*, the mere isolation of the organism is not diagnostic for disease. Supporting information is needed to establish a definitive diagnosis. For many diseases, confirmatory microscopic lesions provide the necessary supportive information to establish a definitive diagnosis. In this study, sections of intestine were critically evaluated to determine whether lesions could be used to support a diagnosis of *C. perf* type A enteritis. Results were equivocal. Microscopic changes in small intestinal were mild, but were on average distinct from controls. Gram-positive rods were noted in both principal and control pigs, although the average score was higher in principals (1.12 vs 0.33). Unfortunately, two issues limit the usefulness of microscopic changes to confirm a diagnosis: 1) a consistently superior sampling location was not identified, and 2) lesions were not diffuse and differences typically only become apparent following examination of multiple sections of intestine. Because lesions were multifocal, mild, and randomly distributed, microscopic examination of intestinal sections did not appear to be of great value in confirming a diagnosis of *C. perfringens* type A enteritis.

Previous studies by our laboratory have suggested that the isolation of a moderate to heavy growth of *C. perf* type A from multiple segments of small intestine is the best indicator of *C. perf* type A enteritis. Results from this study provide further support for this notion as significantly higher levels of *C. perfringens* were isolated from pigs with suspect *C. perfringens* type A pigs enteritis than from apparently healthy controls.

Clostridium perfringens type A enteritis in neonatal swine is a disease purported to be common and economically significant in the breeding herd, yet there are gaps in many aspects of our understanding of this condition. Results of this study provide support for the concept that *C. perfringens* type A is a disease of neonatal swine which results from qualitative and quantitative differences of the intestinal population of *C. perfringens*. A semiquantitative estimate of bacterial growth in culture remains the most useful diagnostic test, while histologic lesions and beta2 toxin detection failed to be of diagnostic value.

Competitive exclusion and vaccination as a means to control *C. difficile* infection.

The effect of competitive exclusion, by a nontoxigenic strain of *C. difficile*, on preweaning performance was to be evaluated by selecting 200 gestating sows/gilts from a candidate herd with CDAD, randomize animals into two groups of 100, dosing piglets with 1 ml of spores prepared from a nontoxigenic strain, and collecting data on number of diarrhea days per group, number of pigs born live/weaned, weaning weights (individual pigs, litter totals, average), and the rate at which *C. difficile* and its toxins were detected in rectal swabs.

The efficacy of vaccination with an experimental CDAD vaccine was to be evaluated. An appropriate dose of vaccine was to be determined by using several different doses of a TcdA-based vaccine in pigs. Once an appropriate dose was established, a candidate farm with a high level of endemic CDAD was to be identified. 100 sows/gilts were to be randomized into 2 groups of 50. They were to be vaccinated twice (4 and 8 weeks before farrowing). Data to be collected would include diarrhea days, pigs born live/weaned, weaning weights (individual pigs, litter totals, average) for all litters. Rectal swabs were to be obtained from 5 pigs per litter at 5 days of age will be examined by bacteriologic culture and toxin testing.

These two objectives could not be completed as the lead investigator (Dr. Yaeger) accepted a new position in a different department. His new responsibilities no longer provided the necessary access to materials or the necessary time to carry out the remaining studies. Dr. Ramirez then took over the project re-directing it to develop a *Clostridium difficile* pig model to be utilized to further investigate the pathogenesis as well a possible intervention steps to mitigate *C. difficile* disease in piglets. Before this study started there were no published models for creating *C. difficile* disease in piglets. It was also the goal of this project to be able to create disease in piglets with an actual *C. difficile* isolate from pigs. After several tries, we were able to take snatched farrowed pigs and inoculate them with *C. difficile* isolates from actual field cases diagnosed with CDAD submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU-VDL). We were successful in achieving our goal as well as were able to identify variations in clinical, gross, and histologic lesions between isolates used. With the development of this model, in future studies we are now able to look at possible interventions with a simple and repeatable way to evaluate the process.

Scientific Abstract:

Published studies report that in piglets less than a week of age with the complaint of diarrhea, *Clostridium difficile* and *Clostridium perfringens* type A were detected in 29-50% and 47-48% of the cases respectively and represent the two most commonly identified agents. This study was intended to: 1. further characterize the effect of *C. difficile* infection on the colon of affected piglets, 2. refine the diagnostics for *Clostridium perfringens* type A enteritis in swine, and 3. Develop a repeatable model for *C. difficile* in pigs. Twelve piglets with suspect CDAD were evaluated to determine the preferred sampling strategy for *C. difficile* diagnostics. Samples were collected from stomach, jejunum, ileum, cecum, 9 segments of colon, and rectum from each pig and submitted for *C. difficile* culture, *C. difficile* toxin ELISA, and microscopic examination. For the purposes of this study, a pig was considered to have *C. perfringens* type A enteritis if the following criteria were met: 1) there was gross evidence of diarrhea, 2) other infectious causes of diarrhea were excluded (*E. coli*, rotavirus, TGE, *C. difficile*, *Salmonella*, *C. perfringens* type C, PRRSV), and 3) a heavy growth of *C. perfringnes* type A was isolated from mid small intestine.

Results indicate that for *C. difficile* diagnostics, cecum and colon remain the preferred sites. A consistently superior sampling location in these sections of large bowel was not identified. All methods (toxin, culture, microscopic lesions) were relatively similar and typically detected 66-77% of affected pigs. Sampling of more than one site from each pig improved the diagnostic sensitivity to approximately 97%. Toxin was detected in 78% of fecal swabs from animals positive for *C. difficile* toxin in colon contents. Results of this study indicate that cases of *C. difficile* infection can be missed if only one sample from the large intestine is evaluated. If two or more samples are evaluated, the sensitivity appears to be quite high (97%). Testing of fecal swabs for *C. diff* toxin may be useful to diagnose *C. diff* issues on a herd basis.

Significantly higher numbers of *C. perf* type A were harvested throughout the intestinal tract of suspect *Clostridium perfringens* type A pigs and a higher percentage of organisms were positive for the beta2 toxin gene, when compared to controls. Two issues limit the usefulness of microscopic changes to confirm a diagnosis: 1) a consistently superior sampling location was not identified, and 2) lesions were not diffuse and differences typically only become apparent following examination of multiple sections of intestine. Because lesions were multifocal, mild, and randomly distributed, microscopic examination of intestinal sections did not appear to be of great value in confirming a diagnosis of *C. perfringens* type A enteritis. A significantly different rate of beta2 toxin detection was not identified in affected pigs compared to control pigs. Results of this study provide support for the concept that *C. perfringens* type A is a disease of neonatal swine which results from qualitative and quantitative differences of the intestinal population of *C. perfringens*. A semiquantitative estimate of bacterial growth in culture remains the most useful diagnostic test, while histologic lesions and beta2 toxin detection failed to be of diagnostic value.

We have also been able to create a repeatable pig model for studying *C. difficile* without having to use antibiotics. This model uses conventional pigs that are snatched farrowed and raised in individual pens. We were also able to create CDAD using both the well know human isolate as well as three additional isolates from Iowa swine field cases. We were also able to demonstrate differences in lesions between these different strains.

Introduction:

In a study entitled "The economic cost of major health challenges in large US swine production systems" (Holtkamp D et. al., AASV proceedings, 2007), *Clostridium perfringens* type A was ranked 3rd and *Clostridium difficile* 12th in terms of the most important health challenges in the breeding herd. Two previous studies in our laboratory demonstrated that in piglets less than a week of age submitted to the Iowa State University Veterinary Diagnostic Laboratory with the complaint of diarrhea, *Clostridium difficile* and *Clostridium perfringens* type A were detected in 29-50% and 47-48% of the cases respectively and represent the two most commonly identified agents.

Clostridium difficile (*C. diff*) has been associated with human antibiotic-related diarrhea and colitis for nearly 25 years. Its importance in humans has prompted detailed studies of the organism, its natural history, and its mechanisms of pathogenesis. In man, *Clostridium difficile*-associated disease (CDAD) is typically a geriatric disease that begins with disruption of the normal colonic flora by antimicrobials or chemotherapeutic agents. Alterations in the competitive microflora provide an open niche for *C. diff* spores to germinate, multiply rapidly, and cause disease through the elaboration of toxins. Disease may present as diarrhea, colitis without pseudomembranes, pseudomembranous colitis, or fulminant colitis.

In man and other species, *Clostridium difficile* is an opportunist. Considering the above described pathogenesis, there are three potential points where this disease could be prevented/controlled: 1) prevent disruption of the normal competitive microflora (ie. eliminate the use of antibiotics), 2) competitive exclusion (ie. eliminate the vacant niche needed for this organism to proliferate) and c) vaccination (vaccinate with a toxoid to neutralize disease-inducing toxins).

Experimental work in hamsters has shown that toxigenic *C. difficile* can be excluded by pre-colonization with nontoxigenic (avirulent) strains of *C. diff*. Results of our preliminary study in pigs were similar.

Parenteral and mucosal immunization with toxoids has been reported in rodents, and protection is apparently associated with high serum levels of toxin-neutralizing antibodies. Mice immunized with the recombinant COOH-terminal portion of TcdA were protected against three lethal doses of TcdA. Vaccination of hamsters protected against death and, to a lesser extent, against diarrhea. We had intended to evaluate both competitive exclusion and vaccination as a means of preventing CDAD in pigs.

Initial outbreaks of CDAD in pigs describe dyspnea, mild abdominal distension, scrotal edema, and diarrhea in young piglets. Ascites (> 50 ml), conspicuous edema of the ascending mesocolon, and hydrothorax were common. Histological lesions included severe submucosal and mesocolonic edema in the ascending colon, with multifocal exudation of mucus, fibrin, and PMN aggregates. In an outbreak in Quebec, mortality of 16% was reported, mainly the result of respiratory distress (due to ascites and severe mesocolonic edema).

Following the initial descriptions of *C. difficile* in swine, several studies were undertaken to determine the prevalence of this disease. These studies demonstrated that *C. diff* toxins are common (29-50%) in the colon of piglets less than a week of age. However, a number of disturbing inconsistencies have been identified, which include: 1) inconsistent clinical signs (the association between toxin and dyspnea, mild abdominal distension, scrotal edema, and diarrhea in young piglets is rare), 2) the detection of toxins in apparently normal healthy pigs, 3) the lack of correlation between initially reported gross lesions (mesocolonic edema) and toxin, and 4) a lack of consistent association with diarrhea.

Too little is known about the minimum standards for establishing a diagnosis of CDAD. It is relatively common for piglets to be toxin positive and lesion negative, or vice versa. This may be due to scattered foci of infection and limited sampling for diagnostic purposes. We propose a systematic and intensive sampling of piglets submitted for diagnosis of enteritis, with the intent of developing more sensitive and specific standards for diagnosis.

Clostridium perfringens type A

    Clostridium perfringens type A enteritis in neonatal swine is a disease purported to be common and economically significant in the breeding herd. For a disease believed to be of considerable importance, information on the pathogenesis, a reliable experimental model, and a gold standard for diagnostics are all lacking.

    Due to frequent lack of microscopic lesions, the occurrence of *C. perf* type A as normal flora, and the isolation of beta2-toxin positive strains from normal animals, the diagnostic criteria are viewed by many as equivocal. Genotyping of *Clostridium perfringes* type A isolates from young pigs with diarrhea demonstrate that this disease is likely associated with strains of *Clostridium perfringens* that are positive for the beta2 toxin gene. A major aim of this study was to critically assess *C. perf* type A diagnostics and evaluate assays for beta2 toxin as a potential tool for the diagnosis of this disease.

Clostridium difficile has been demonstrated as a potential pathogen in pigs, with diagnostic criteria that include clinical signs, lesions, toxin detection, and isolation of the offending organism. Recent attention has been directed toward pork products as a possible source of human infections. There is need for a reliable animal model to evaluate interventions for mitigation of infections, disease, and carriage with greater confidence.

Objectives:
1. To further characterize the effect of *C. difficile* infection on the colon of affected piglets
2. To refine the diagnostics of Clostridium perfringens type A enteritis in swine.
3. To evaluate competitive exclusion as a means for preventing Clostridium difficile associated disease (CDAD)
4. To evaluate an experimental human CDAD vaccine, on which a pig product can be based.

After Dr. Ramirez took over the project, the objectives changed for the balance of the project to start with priority 1 below and work until successful or funds run out. If successful and still have funds will then move to priority 2.
1. Development of a reliable challenge model for suckling piglets (Primary goal) – See study design below
    a. Will look at replicating existing models from mice, hamsters, and prairie dogs into swine.
    b. We will need to evaluate several antibiotics as possible means for achieving an environment conducive for C. difficile disease. This information is quite valuable in helping establish the association of C. difficile disease and antibiotic usage.
    c. We will be looking at clinical signs, histologic lesions, and toxin detection for the different schemes evaluated.
    d. Since this has not been done before, we are not sure how many tries it will take to accomplish this.
    e. This will require multiple replications of several protocols.
2. Use of this model to evaluate interventions (Based on time and funds available)
    a. Carriage model using conventional pigs to establish duration and magnitude (TOP PRIORITY)
    b. Competitive exclusion
    c. experimental vaccines

Materials & Methods:

Clostridium difficile:

12 piglets with suspect CDAD were evaluated to determine the preferred sampling strategy for *C. difficile* diagnostics. Samples were collected from stomach, jejunum, ileum, cecum, 9 segments of colon, and rectum from each pig and submitted for *C. difficile* culture, *C. difficile* toxin ELISA, and microscopic examination.   Toxin was quantified by ToxA/B enzyme immunoassay, *C. difficile* was semi-quantified by culture, and lesions were scored in terms of severity, the presence of neutrophils, and/or erosions/ulcerations. Data was analyzed to determine segments in which the correlation of these factors was the strongest.

Clostridium perfringens type A:

For the purposes of this study, a pig was considered to have *C. perfringens* type A enteritis if the following criteria were met: 1) there was gross evidence of diarrhea, 2) other infectious causes of diarrhea were excluded (*E. coli*, rotavirus, TGE, *C. difficile*, *Salmonella*, *C. perfringens* type C, PRRSV), and 3) a heavy growth of *C. perfringnes* type A was isolated from mid small intestine. The following samples were collected from 10 pigs fulfilling these criteria and from 10 apparently healthy control pigs. Swabs were collected from the following sites for semi-quantitative *C. perfringens* culture: stomach, duodenum, small intestine at 10 cm intervals, ileum, and rectum. Contents were collected from each segment for beta2-toxin testing, and fixed tissue was processed for microscopic examination. Ten *C. perfringens* isolates from each segment of bowel were analyzed by pulse filed gel electrophoresis. Beta2 toxin analysis was performed with a dot blot technique. Intestinal contents were passed through a nitrocellulose membrane using a 96 well dot blot apparatus. The membrane traps all larger proteins. A primary antibody directed against beta2 toxin was added to the membrane. Wash steps removed any unbound antibody. A secondary antibody with attached enzyme was added. The presence of toxin was indicated by an enzymatic color change Serial dilutions of beta2 toxin (1 ug/ml, 0.5, 0.25, 0.12, 0.6, 0.03, 0.015, 0.008, and 0.004 ug/ml) were used to evaluate the sensitivity of the test. A distinct color change was observed down to 0.004 ug/ml beta2 toxin.

Effect of competitive exclusion, by a nontoxigenic strain of C. difficile, on preweaning performance. We were to select 200 gestating sows/gilts from a candidate herd with CDAD and randomize animals into two groups of 100. Piglets were to be dosed with 1 ml of spores prepared from a nontoxigenic strain. Collected data would include the number of diarrhea days, number of pigs born live/weaned, and weaning weights (individual pigs, litter totals, average). Rectal swabs were to be obtained from 5 pigs per litter at 5 days of age and examined by bacteriologic culture and toxin testing.

Efficacy of vaccination with an experimental CDAD vaccine. We proposed to evaluate a TcdA-based vaccine it in pigs. We were to perform a dose titration in growing pigs. Pigs (n = 24) ~ 3 months of age were to be randomized into 4 groups of 6 and vaccinated twice (with doses of 50, 100, 150, and 200 µg per vaccination) at a 4 week interval. They were to be bled before the first and second vaccinations and two weeks after the second. Sera was to be examined for neutralizing antibodies. On a candidate farm with a high level of endemic CDAD, we were to select 100 sows/gilts and randomize them to 2 groups of 50. They were to be vaccinated twice (4 and 8 weeks before farrowing) with the most appropriate dose determined above. Serum for neutralization tests were to be obtained as near as possible to farrowing. Data collected would include diarrhea days, pigs born live/weaned, weaning weights (individual pigs, litter totals, average) for all litters. Rectal swabs were to be obtained from 5 pigs per litter at 5 days of age and examined by bacteriologic culture and toxin testing.

Clostridium difficile pig model.
Study design (Euthanasia schedule) for each replicate:

	0 hrs.	24 hrs.	48 hrs.	72 hrs.
Challenge Group	0	2	2	2 or 3
Controls	0	1	1	1

Snatched farrowed pigs were obtained form a commercial farm producing PRRS negative pigs. All study pig where then transported to our BSL-2 University facility and housed individually in specially designed boxes with raised flooring. All pigs were housed in the same air space. Room temperature was maintained at 29°C and supplemental heat lamps were used. Pigs were fed a commercial milk replacer. Piglets were randomly assigned to one negative control group (n=3) and two different treatment groups (n=6 or 7 for each treatment). Each treatment group received one of four different *C. difficile* isolates (one human reference strain and three field isolates from ISU-VDL). This process was replicated three different times to evaluate variations in the model as well as strain variations. At approximately 5 hours after birth, piglets in treatment groups were inoculated with approximately 2X10^{9} spores/ml of the respective isolate via a gastric tube. Pigs from each group including the negative controls were randomly selected for necropsy at 24, 48, and 72 hours post inoculation. Gross observations were scored as described by Yaeger et al 2007 including body condition, hydration status, perineal fecal staining, colonic contents consistency, and mesocolonic edema. Colonic and cecal content were collected for bacterial isolation including *C. difficile* selective agar. Pooled colon and cecum contents were assayed for *C. difficile* toxins using a commercially available toxin ELISA kit according to manufacturer instructions and analyzed on a microplate reader assigning a score of) (no toxin production) to 4+ (marked toxin production). Histologic sections of the ileum, jejunum, descending colon, cecum and cross section of the spiral colon were evaluated by a diagnostic pathologist.

Results:

Clostridium difficile:
12 piglets with suspect CDAD were evaluated to determine the preferred sampling strategy for *C. difficile* diagnostics. Samples were collected from stomach, jejunum, ileum, cecum, 9 segments of colon, and rectum from each pig and were submitted for *C. difficile* culture, *C. difficile* toxin ELISA, and microscopic examination. Of the 12 pigs evaluated, *C. difficile* toxin was detected in at least 1 segment from 9 animals.

GI segment	*C. difficile* culture (# of positive pigs)	*C. diff* toxin ELISA (# of positive pigs)	Lesions (# of positive pigs)
Stomach	4	0	0
Jejunum	6	0	0
Ileum	7	0	0
Cecum	7	6	6
Colon 1	6	6	6
Colon 2	7	6	7
Colon 3	6	7	6
Colon 4	6	6	6
Colon 5	7	6	7
Colon 6	7	5	5
Colon 7	7	6	7
Colon 8	6	7	6
Colon 9	6	6	6
Rectum	7	6	--
Rectal swab	6	7	--

Clostridium difficile was isolated from stomach, small intestine and colon. A significant difference was not identified in the rate at which *C. difficile* was isolated from various segments of the gastrointestinal tract. Neither *C. difficile* toxin nor lesions were identified in stomach. *C. difficile* was isolated from approximately 50% of small intestinal segments, yet *C. difficile* toxin was not detected in small intestinal contents and microscopic lesions were not observed in the small intestine.

     There was a statistically significant association between *C. difficile* isolation, detection of *C. difficile* toxins, and lesions in both the cecum and colon ($p \leq 0.05$), but not in the small intestine or stomach. There were no statistically significant differences between large intestinal sites in regards to *C. difficile* isolation, lesions, or toxin ELISA results. A consistently superior site from which to collect samples for *C. difficile* diagnostics was not identified. Toxin was detected in 78% of fecal swabs from animals positive for *C. difficile* toxin in colon contents.

Clostridium perfringens type A:
     Culture results, lesions and beta2 toxin levels were compared between 10 suspect *C. perf* type A pigs and 10 apparently healthy control pigs. In pigs with suspect *C. perf* type A disease, the average level of bacterial isolation was significantly higher (2.39 ± 0.4 vs 0.64 ± 0.31; $p < 0.01$) than the controls. *C. perfringens* type A was not consistently isolated at higher levels from any single segment of bowel. A trend towards higher or lower levels of isolation in proximal or distal segments of small bowel was not identified.

     Clostridium perfringens type A was commonly isolated from the stomach. The culture level from the stomach of suspect *Clos perf* type A pigs was 2.58. 10 isolates from each segment of bowel were evaluated for the beta2-toxin gene (CPB2). The average percent of isolates producing CPB2 was higher in principals than in controls (87.2% vs 53.8%), but the difference was not significant, due to substantial variability among control samples. Nearly all isolates from stomachs of principals and controls were CPB2-positive.

     Suspect *Clos perf* type A pigs had higher average lesion scores throughout small intestine (average score 0.52 ± 0.22) compared to controls (average score 0), but variation in lesion scores from proximal to distal small intestine was not significant. Gram-positive rods were noted in both principal and control pigs. The average score was higher in suspect *Clostridium perfringens* type A pigs (1.12 vs 0.33). These organisms were seen in only three segments of small intestine from all control pigs, but this variability eliminated statistically-significant differences between groups.

     Dot blots for beta2 toxin were positive in 23/300 (7.7%) of suspect *C. perf* type A pig samples and 19/300 (6.3%) of control pig samples. A significant difference was not identified.

Partial results of Beta2 toxin dot blot analysis of suspect *C. perfringens* type A pigs and apparently healthy control pigs.

Animal ID	Site 1	Site 2	Site 3	Site 4	Site 5	Site 6	Site 7	Site 8
Case	neg	neg	neg	neg	neg	neg	neg	neg
Case	neg	neg	neg	neg	neg	neg	neg	neg
Case	POS +3	POS +1	neg	POS +1	POS +1	POS +2	POS +3	POS +3
Case	neg	neg	neg	neg	neg	neg	neg	neg
Case	neg	neg	neg	neg	neg	neg	neg	
Case	neg	neg	neg	neg		neg	neg	neg
Case	neg	neg	neg	neg	neg	neg	neg	neg
Case	neg	neg	neg	neg	neg	POS wk	neg	neg
Case	neg	neg	neg	neg	neg	neg	neg	neg
Case	neg	POS wk	neg	neg	neg		neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg
Control	POS wk	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	POS wk	POS wk	POS wk	POS wk	POS wk
Control	neg	POS wk	POS wk	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg		neg
Control	neg	POS wk	POS wk	neg	POS wk	neg	POS wk	neg

Animal ID	Site 9	Site 10	Site 11	Site 12	Site 13	Site 14	Site 15
Case	neg	neg	neg	neg	neg	neg	neg
Case	neg	neg	neg	neg	neg	neg	neg
Case	POS +3	POS +3	POS +2	POS +2	POS +2	POS +1	Pos wk
Case	neg	neg	neg	neg	POS wk	neg	neg
Case	neg		neg	neg	neg	neg	neg
Case	neg	neg	neg	neg		neg	neg
Case	POS wk	POS wk	POS wk	POS wk	POS wk	POS wk	neg
Case	neg	neg	neg	neg	neg	neg	neg
Case	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	POS wk	neg
Control	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg		neg
Control	neg	neg	neg	neg	neg	neg	neg
Control	neg	neg	neg	neg	neg	neg	neg
Control		neg	POS +2	neg	neg		neg
Control	neg	neg	neg	POS wk			neg
Control	neg	neg	neg	neg	neg	neg	neg
Control	POS wk	neg	POS wk	POS wk	POS wk	neg	neg

In the *C. difficile* model development project, once the model was refined we were able to run two more study groups through the model. In the last two studies involving 32 pigs, *Clostridium difficile* was isolated from the colon of all pigs at necropsy. Mesocolonic edema was more common at 48 and 72hpi (hours post inoculation) with some control pigs exhibiting mesocolonic edema at 24hpi. Overall, mesocolonic edema was present in 73% of the challenged pigs compared to 33% of the controls. There was quite a bit of variation in gross and microscopic lesions between the different isolates as well as at the different times. The standard human isolate appears to have greater lesions at 72 hours compared to 24 hours. On the other hand, all 3 pig isolate had more severe lesions at 24 hours. Only 57.6% of the challenged pigs had clinical signs of diarrhea.

Isolate	Toxinotype	Origin
JGS6125	Novel	Human
13912-1	V	Pig in Iowa
02792-1	V	Pig in Iowa
15454-1	Novel	Pig in Iowa

Toxin production also seemed to vary between isolates and time frames. The detection of toxin production was different between colon and rectum sampling with the colon usually having higher toxin scores.

Group	Mesocolonic edema	Toxin	Diarrhea
Challenge (n=26)	19 (73%)	18 (69.2%)	15 (57.6%)
Control (n=6)	2 (33.3%)	3 (50%)	2 (33.3%)

Histologic lesions were characterized by mild to severe neutrophils infiltration especially in the colon as well mild to moderate goblet cell loss. These intestinal lesions were found in 70 – 80% of the challenged pigs both at 24 and 72hpi.

Fund were used up creating repetitions of our pig model using swine specific strains so we were unable to work on part 2 of the redirected objectives.

Discussion:

Clostridium perfringens type A:
    For a disease believed to be of considerable importance, information on the pathogenesis, a reliable experimental model, and a gold standard for diagnostics are all lacking. *Clostridium perfringens* type A enteritis in neonatal swine remains largely an enigma.
    We have proposed the following pathogenesis for *Clostridium perfringens* type A enteritis, based on these and previous results. The stomach acts as a fermentation vessel for the proliferation of *C. perfringens* with continuous seeding of the intestine resulting in high levels of *C. perfringens* throughout the entire small bowel. The lack of consistent villus atrophy or necrosis would suggest that released beta2 toxin results in a secretory diarrhea.
    The results of this study provide support for parts of this proposed pathogenesis. The concept that *C. perfringens* type A enteritis is due to quantitative and qualitative changes in the intestinal population of *Clostridium perfringens* in supported by the higher numbers of *C. perf* type A harvested throughout the intestinal tract of suspect *Clostridium perfringens* type A pigs and the higher percentage of organisms positive for the beta2 toxin gene. The isolation of significantly higher numbers of beta2 toxin gene positive isolates from the stomach supports the concept that these quantitative and qualitative differences begin in the stomach.

Diagnostics for *C. perf* type A remain a central issue. For discussion purposes, diagnostics will be divided into three issues: 1) beta2 toxin detection, 2) microscopic changes, and 3) culture results.

Based on genotyping results, which have associate *C. perfringens* type A enteritis with beta2-toxin gene positive strains of the organism, it was proposed that a test measuring beta2 toxin in intestinal contents could represent a useful diagnostic tool. This test would potentially have the advantage of detecting both the causative principal (beta2 toxin) and providing information on the level of toxin present, as a threshold for clinical significance was considered likely.

A statistically significant increased rate of beta2 toxin detection was not identified in affected pigs compared to control pigs. It does not appear that analysis for beta2 toxin will prove to be a useful test for the diagnosis of *C. perf* A enteritis. Overall results also bring into question the specific role of the beta2 toxin in the pathogenesis of this disease. The results from one pig were intriguing. The beta2 toxin results mirrored culture results in that high levels of bacterial growth were isolated from all segments and beta2 toxin was identified in all segments of small intestine. Had this been a consistent finding, this wound have supported the potential diagnostic utility of beta2 toxin testing. Unfortunately, in the pigs examined, this result was the exception rather than the rule.

For ubiquitous organisms, such as *E. coli* and *Clostridium perfringens*, the mere isolation of the organism is not diagnostic for disease. Supporting information is needed to establish a definitive diagnosis. For *E. coli*, histopathology, with the identification of intestinal colonization by short, rod-shaped bacteria, often provides the supporting information necessary to establish a definitive role for *E. coli* in the disease process. In this study, sections of intestine were critically evaluated to determine whether lesions could be used to support a diagnosis of *C. perf* type A enteritis. Results were equivocal. Microscopic changes in small intestinal were mild, but were on average distinct from controls. Gram-positive rods were noted in both principal and control pigs, but the average score was higher in principals (1.12 vs 0.33). Unfortunately, two issues limit the usefulness of microscopic changes to confirm a diagnosis: 1) a consistently superior sampling location was not identified, and 2) lesions were not diffuse and differences typically only become apparent following examination of multiple sections of intestine. Because lesions were multifocal, mild, and randomly distributed, microscopic examination of intestinal sections did not appear to be of great value in confirming a diagnosis of *C. perfringens* type A enteritis.

Previous studies by our laboratory have suggested that the isolation of a moderate to heavy growth of *C. perf* type A from multiple segments of small intestine is the best indicator of *C. perf* type A enteritis. Results from this study provide further support for this notion as significantly higher levels of *C. perfringens* are isolated from pigs with suspect *C. perfringens* type A pigs enteritis than from apparently healthy controls.

Clostridium perfringens type A enteritis in neonatal swine is a disease purported to be common and economically significant in the breeding herd, yet there are gaps in many aspects of our understanding of this condition. Results of this study provide support for the concept that *C. perfringens* type A is a disease of neonatal swine which results from qualitative and quantitative differences of the intestinal population of *C. perfringens*. A semiquantitative estimate of bacterial growth in culture remains the most useful diagnostic test, while histologic lesions and beta2 toxin detection failed to be of significant diagnostic value.

Clostridium difficile:
The presence of *C. difficile* toxins in the colon of a high percentage (29-50%) of piglets less than a week of age has been well-documented. However, there are a great many disturbing inconsistencies when the presence of toxin is correlated with clinical signs, gross and microscopic lesions. Initial studies described an association between *C. difficile* toxins and dyspnea, mild abdominal distension, scrotal edema, and diarrhea in young piglets. Subsequent studies have generally failed to associate toxin with any of these clinical changes and propose that pigs with *C. difficile* toxin are more likely to have normal or

constipated feces. Initial studies also described a strong association with grossly visible mesocolonic edema, which was found to be of limited predictive value in subsequent studies.

Too little is known about the minimum standards for establishing a diagnosis of CDAD. It is relatively common for piglets to be toxin positive and lesion negative, or vice versa. This may be due to scattered foci of infection and limited sampling for diagnostic purposes. A systematic and intensive sampling of piglets submitted for diagnosis of enteritis was undertaken in an attempt to refine the approach for *C. diff* diagnostics and to determine if reported inconsistencies are due to the diagnostic approach or other factors.

Study results support the following conclusions. Cecum and colon remain the preferred sites for *C. difficile* diagnostics. A consistently superior sampling location in the large bowel was not identified. All methods (toxin, culture, microscopic lesions) were relatively similar and typically detected 66-77% of affected pigs. Sampling of more than one site from each pig improved the diagnostic sensitivity to approximately 97%.

The use of fecal swabs as a tool for ante mortem testing for *C. difficile* was evaluated. If sufficiently sensitive, this method would be preferred because it would eliminate the need to sacrifice piglets. Toxin was detected in 78% of fecal swabs from animals positive for *C. difficile* toxin in at least one segment of large bowel. This level of sensitivity was not considered to be adequate for individual animal diagnostics, but testing of fecal swabs for *C. diff* toxin may be a useful way to diagnose *C. difficile* involvement on a herd basis.

Results of this study indicate that cases of *C. difficile* infection can be missed if only one sample from the large intestine is evaluated. If two or more samples are evaluated, the sensitivity appears to be quite high (97%).

Previous studies have described a number of inconsistencies in *C. difficile* diagnostics.   Despite a strong association between *C. difficile* toxins and colitis in a number of studies, some pigs with high levels of toxin do not have microscopic lesions in their cecum or colon, and do not have diarrhea. Though not apparent from the raw data above, similar inconsistencies were identified in this study. Toxin was not detected in 20% of large intestinal sites that were positive by culture. *C. difficile* toxin was detected in 16% of culture negative sites. Typhlitis was observed in 72% of toxin positive samples and 23% of toxin negative samples. Colitis was detected in 75% of toxin positive samples and 25% of toxin negative samples. When all large intestinal sites from one animal were considered, 87% of animals with lesions in the cecum or colon were toxin positive. It does not appear that sampling issues can account for all of the inconsistencies and more work needs to be done to better characterize the significance of this organism and its toxins in the large intestine of young pigs.

Objective 3 & 4 could not be met. The lead investigator (Dr. Yaeger) accepted a new position in a different department. His new responsibilities no longer provided the necessary access to materials or the necessary time to carry out the remaining objectives.

  In the redirection of the last part of the project, our model was successful in generating *C. difficile* disease in newborn piglets. We were able to inoculate pigs, cause lesions (gross and histologic) characteristic of *C. difficile* infection. There was some slight contamination of our control pigs, so future models would necessitate the housing of control in a separate room. *Clostridium difficile* is a common pathogen found in all pigs. The snatch farrowing process, environmental contamination (including possible aerosol), or personnel cross contamination could have occurred.

Our findings identify differences in strains on lesions associated with *C. difficile*. Our model is the first one to utilize actual swine isolates to generate *C. difficile* disease in pigs. This is also the first study to demonstrate *C. difficile* lesions without the use of antibiotics. This new model is repeatable and easy to do without needing special pig sources or incurring the high expense of CDCD pigs. This is a very important step in paving the way to further research this interesting pathogen.