RESEARCH REPORT
SWINE HEALTH

Title: Effect of age, dose and antibiotic therapy on the development of neonatal Clostridium difficile disease - **NPB #10-058**

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Industry Summary

Neonatal piglet diarrhea is associated with increased pre-weaning mortality, poor growth rates, and variation in piglet weight at weaning. Within the last decade, neonatal diarrhea has been increasingly associated with the presence of *Clostridium perfringens* type A and/or *Clostridium difficile*. Clostridium difficile-associated disease (CdAD) is manifested as mild to severe colitis in humans, horses, piglets as well as other animal species. Many risk factors are thought to contribute to CdAD in piglets, including administration of antimicrobials at processing, piglet age, or overall hygiene in farrowing crates (environmental load; dose). However, this anecdotal data has not been investigated. The objectives of this research was to: 1) evaluate the consistency and severity of disease lesions in piglets challenged at different bacterial doses, to (2) evaluate the use of antimicrobials as a contributing risk factor in the development of disease, to (3) provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets, and (4) try to develop and validate an immunohistochemistry (IHC) test using commercially available antibodies specific for toxin A and toxin B of *Clostridium difficile* to determine if one or both toxins are associated with lesions.

Three separate pig experiments were conducted to answer the study objectives. Neonatal pigs were snatch-farrowed from a commercial sow farm and received 10 ml of pooled colostrum from the farm of origin via gastric lavage. Piglets were then transported to Iowa State University and individual housed for experimental *Clostridium difficile* inoculation. Three days post-challenge, pigs with euthanized for sample collection. Combined results of the three animal studies indicate that *Clostridium difficile*

dosage appears to be an important factor that influences the appearance and severity of lesions, 10 day-old pigs can develop disease associated with *Clostridium difficile*, and antibiotic administration following inoculation did not significant increase disease or lesion severity. This data suggests that good sanitation may reduce CdAD in young, older piglets can be affected by the bacteria and antimicrobial therapy at processing does not increase disease severity.

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Scientific abstract

Neonatal piglet diarrhea is associated with increased pre-weaning mortality, poor growth rates, and variation in piglet weight at weaning. *Clostridium difficile* is a known factor contributing to diarrhea at this age, yet risk factors associated with *C. difficile* disease in neonatal piglets is unknown. The objectives of this study were to: (1) evaluate the use of antibiotics as a contributing risk factor in 1-day-old piglets, to (2) evaluate the consistency and severity of disease lesions in piglets challenged at different bacterial doses, and to (3) provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets. One hundred and eleven conventional neonatal pigs were snatched farrowed and divided into three separate experiments: DOSAGE, ANTIMICROBIAL, and AGE. In the DOSAGE experiment, 40 1-day-old-piglets were sham inoculated or challenged with varying amounts of C. difficile heat shock spores and euthanized 72 hrs. post infection. Results indicate a clear trend for disease development as bacterial amounts increase. In the ANTIMICROBIAL experiment, 39 1-day-old-piglets were challenged and treated with one or four different antibiotics 16hrs later. No significant difference for disease development was found. Thirty-three 10-day-piglets were given varying doses of *C. difficile* in the AGE experiment. Disease and lesions were reproduced in this age of piglet. Combined results from the three experiments indicate that *C. difficile* dosage appears to be an important factor that influences the appearance and severity of lesions, 10 day-old pigs can develop disease associated with *Clostridium difficile*, and antibiotic administration following inoculation may not be a substantial factor for disease in neonatal piglets.

Introduction

Clostridium difficile is a Gram positive, anaerobic, spore-forming bacterium. The bacterium was first described in 1935 as part of the neonate intestinal flora;[1] however, it was not until 1978 that the bacterium was associated with human disease.[2] *Clostridium difficile* infection (CDI) is characterized by mild to severe diarrhea and has been described in several species including humans, pigs, horses, non-human primates, rabbits, rats, domestic dogs and domestic cats.[3,4,5,6,7] In humans, CDI is now the major cause of antibiotic-associated diarrhea in several countries worldwide.[8,9,10,11,12,13]

The incidence of CDI has been steadily increasing in veterinary medicine. The majority of cases are associated with the disequilibrium of commensal intestinal flora. Neonates and animals treated with select antimicrobials are most commonly described. [14] [15]. Antimicrobials eliminate susceptible microorganisms present in the intestine, allowing strains of *C. difficile* to overgrow because of a lack of competition from the microbiota. Results from a study where hamsters were injected daily with clindamycin showed that susceptible microorganisms were not able to colonize the intestines for several days after the last dose of the antibiotic. Conversely, the bacterial species which were resistant to that drug, were able to multiply and rapidly colonize.[14]

CDI in piglets is associated with large bowel inflammation with the potential for pseudomembranous formation. Disease can also manifest systemically resulting in ascites, pleural effusion, hepatic abscess, renal dysfunction, and acute respiratory distress.[13] The mechanism by which the microorganism causes systemic disease is not completely understood, but exotoxin generation and profile is believed to be a major virulence factor. Toxin A, toxin B and binary toxin are known products of certain strains of *C. difficile*. Toxin A and/or toxin B are large polypeptide and are believed to be the essential virulence factors associated with disease development.[16,17] Toxin A is known for entero-toxicity while toxin B is a potent cytotoxin *in vitro,*[10] however some researchers have failed to report these effects *in vivo* unless prior cellular damage has occurred.

In the field, C. difficile intestinal colonization occurs within the first hours of life in the neonatal pig, and nearly one hundred percent of piglets in some herds are colonized within 48 hours of life.[5] In contrast to colonization, CDI doesn't affect all piglets within a herd or litter, but will generally manifest as mild to severe diarrhea in 1-5 day-old piglets. Neonatal piglets are highly susceptible to the toxins' affects as the intestinal microflora is not fully established. This establishes piglets as a good model for human studies but also presents a serious problem in the swine industry.

Many risk factors are thought to contribute to CDI, including administration of antimicrobials, dose, toxin-type and animal age. However, there is a lack of scientific knowledge regarding these aforementioned risk factors in swine. The objectives of this study were to: (1) evaluate the consistency and severity of disease lesions in piglets challenged at different bacterial doses, to (2) evaluate the use of

antimicrobials as a contributing risk factor in the development of disease, and to (3) provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets.

Objectives

- Determine the effect of dose of *Clostridium difficile* on the severity of clinical disease and development of lesions when inoculated in to 1 day old and 10 day old piglets.
- Determine the effect of antibiotic therapy on severity or incidence of clinical disease and lesions when given 24 hrs. after *Clostridium difficile* infection
- Develop and validate an immunohistochemistry (IHC) test using commercially available antibodies specific for toxin A and toxin B of *Clostridium difficile* to determine if one or both toxins are associated with lesions
- Evaluate the application of the IHC test as a rapid, sensitive, and economical test to diagnose *Clostridium difficile* associated diarrhea in field specimens presented to the VDL

Materials & Methods

Animals

One hundred and eleven conventional neonatal pigs were procured from a 2,500 head sow farm located in Central Iowa. At the farm of origin, selected sows were monitored for signs of parturition by research personnel, peri-vulvar and peri-anal regions were carefully cleaned to remove organic debris with disinfecting wipes (Clorox® Disinfecting Wipes, The Clorox Company, Oakland, CA), and a surgical drape was placed in the farrowing crate behind the sow as a barrier to the surrounding environment. Sows were allowed to farrow naturally. Immediately following delivery, individual piglets were snatched, dried, and placed in plastic totes under heat lamps. Umbilical cords were clamped, cut, and sprayed with gentle iodine.

Mammary glands of farrowing sows were cleaned with disinfectant wipes (Clorox® Disinfecting Wipes, The Clorox Company Oakland, CA) and colostrum was manually collected in 50 ml centrifuge tubes. Equal amounts of colostrum from each sow were combined into a plastic bag and agitated. All neonatal pigs received 10 ml of colostrum mixed with 20 ml of commercial puppy milk replacement (ESBILAC, Hampshire, IL) in the same 60 ml syringe. The 30 ml mixture was delivered via an oral-gastric tube consisting of an eight-gauge rubber French catheter. Pigs were then transported to a BSL-2 animal facility at Iowa State University.

Serum from all procured neonatal pigs was negative for porcine reproductive and respiratory virus nucleic acid by PCR. Serum was analyzed for PRRSV nucleic acids using a licensed real time PCR assay (Applied Biosystems, CA, USA).

Housing

Piglets were housed in one of six identical raised plastic tubs partitioned into eight individual pens (approximately 0.7 x 0.7 m) with clear solid plastic dividing walls. Prior to piglet arrival, rooms and plastic tubs were cleaned with total removal of organic material and disinfected with 2% potassium peroxymonosulfate (Virkon® S, DuPont; Wilmington, DE) for a 4 hour period to effectively eliminate environmental vegetative cells and spores (Lawley et al., 2009a). Room temperature was maintained at 29°C and heat lamps were suspended over the tubs to keep the immediate piglet environment at or around 35°C. All challenged pigs were housed in the same room and airspace. Negative control piglets were housed in a separate room. Pigs were bowl-fed 60 ml of commercial milk replacer three times daily for the duration of the study period. Feedings were spaced approximately seven to nine hours apart.

Experimental Design.

Three separate experiments were completed. Each experiment was a specific objective to be investigated: bacterial dosage was evaluated in experiment 1 (DOSAGE), antimicrobial usage and the development of disease in experiment 2 (ANTIMICROBIAL), and the affected of piglet age in experiment 3 (AGE). In each experiment, pigs were randomly allocated into four (DOSAGE and AGE) or five groups (ANTIMICROBIAL) using several random number iterations in Microsoft Excel®. Table 1 summarizes the experimental design for all three experiments. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee (#9-10-7014-S).

In the DOSAGE experiment, three different quantities of heat shocked Clostridium difficile spores were inoculated. Group 1 received sham material and groups 2, 3, and 4 received $2x10^3$, $2x10^6$, $2x10^9$ C. difficile/ml, respectively. For the ANTIMICROBIAL experiment, all groups were challenged with $2x10^6$ C. difficile/ml. Twelve hours following C. difficile challenge, groups 6, 7, 8, and 9 were administered a different antibiotic. The AGE experiment utilized the same protocol for piglet collection. Piglets were kept for 10 days and then challenged with different concentrations $2x10^3$, $2x10^6$, $2x10^9$ C. difficile/ml (Table 1). Piglets were euthanized 72 hours after challenge in all experiments.

Inoculum.

C. difficile isolate ISU-15454-1, was used for all experiments. This isolate originated from a field case of neonatal diarrhea in 3-6 day-old piglets. High levels of toxin (4+) were detected by ELISA (C. DIFFICILE TOX A/B II™, Balcksburg, VA) from the clinically affected piglets. Isolate 15454-1 is a ribotype 078, toxinotype V, and contains both toxin A and toxin B gene sequences. The isolate was stored at -80°C until culture preparation.

The C. difficile isolate was plated on anaerobic brain heart infusion agar with 0.5% yeast extract and 0.05% L-cysteine (BHI-CYE) for 7 days in an anaerobic environment. Culture lawn was harvested with 5 ml of pH 7.2 phosphate-buffered saline and pelleted by centrifugation for 20 minutes. The pellet was washed with 1M KCl:0.5M NaCl, centrifuged, and suspended in 25 ml 50mM Tris-HCl (pH 7.2) with 10 mg lysozyme per ml. Following 1 hour incubation at 37°C, material was washed 3 times with 25 ml sterile nanopure water, and centrifuged. Spores were counted using a hemocytometer, and the final concentration was adjusted to $2x10^9$ spores/ml in sterile nanopure water. The final spore product was stored at 4°C until inoculation day.

Directly prior to piglet challenge, spore product was tittered with sterile nanopure water for dose as indicated in table 1. Spores were heat-shocked at 80°C for 10 min in a water bath. BHI broth with 0.1% taurocholic acid and 5% fetal bovine serum was added to the heated spore suspension at a concentration of 25% v/v and incubated 1 hour at 37°C (Braun et al., 1996; Kato et al., 1998; Rupnik et al., 1998).

Inoculation.

All piglets were intragastrically inoculated using an eight-gauge rubber French catheter as an oral-gastric tube. Inoculation occurred approximately hours after birth in the DOSAGE and ANTIMICROBIAL experiments, and 10 days for the AGE experiment. The negative control groups in the DOSAGE and AGE experiments were given 1.25 ml of sterile nanopure water, and then flushed with 20 ml of milk replacement. For all inoculated groups in the DOSAGE, ANTIMICORBIAL, and AGE experiments, 1.25 ml of challenge preparation containing heat-shocked Clostridium difficile spores was given followed by 20 ml of milk replacement. Pigs were allowed access to milk replacement (via bowls) immediately after administration of challenge.

Antimicrobials.

Sixteen hours post-inoculation, select groups in the ANTIMICROBIAL experiment (table 1) were intra-muscular administered with an injectable antibiotic commonly used in the swine industry. The

subsequent antibiotics were used: Lincomycin, Ceftiofur (Excede®, Pfizer Animal Health), Tylosin, and Tulathrommcin (Draxxin®, Pfizer Animal Health). All antibiotics were given at labeled dose based on body weight.

Necropsy.

Piglets from all experiments were monitored for 72 hours post-challenge and then euthanized by an intravenous overdose of pentobarbital. Gross observations at necropsy included 1) body condition, 2) dehydration status, 3) perineal fecal staining, 5) consistency of colonic contents, 6) mesocolonic edema, and the presence of 7) visible colonic luminal necrosis and were scored independently in a blinded fashion as previously described (Yaeger et al., 2007).

Sample collection

Immediately prior to inoculation rectal swabs were taken from all pig. At necropsy, fresh and formalin fixed tissues were collected with flamed instruments soaked in 70% alcohol and included: ileum, jejunum, descending colon, cecum, and a cross section of spiral colon containing 4-5 loops. Colonic and cecal contents were collected in a sterile plastic cup. A luminal swab of the ileum was also taken.

Toxin detection and culture.

Rectal swabs collected prior to inoculation and pooled colon and cecum contents retrieved at necropsy were assayed for C. difficile toxins with a commercially available toxin ELISA kit (C. DIFFICILE TOX A/B II™, Balcksburg, VA) used according to manufacturer instructions and analyzed on a microplate reader to semi quantitatively grade the amount of toxin from 0 (no toxin detection) to 4+ (marked toxin detection) as indicated by the manufacturer. Toxin ELISA was performed within four hours following sample collection.

Pooled large intestinal contents and mucosal scrapings from two sections of fresh spiral colon, approximately three to four cm length, were combined and cultured on Clostridium difficile selective agar (CDSA) ; both direct and following a 30 minute room temperature incubation in 0.5 ml absolute ethanol. All plates were incubated at 37°C for 48 hours in an anaerobic chamber. C. difficile growth following incubation was semi-quantitatively scored in a blinded manner by a veterinary microbiologist as follow: 0 = no growth, 1 = few colonies, 2 = low numbers of colonies, 3 = moderate growth and 4= high growth. Rectal swabs collected prior to inoculation from all pigs were also cultured as described above.

Luminal swabs from the small intestine were tested by routine aerobic and anaerobic culture methods to test for Salmonella spp, Escherichia coli, and Clostridium perfringens. Genotyping for Escherichia coli, and Clostridium perfringens were performed according to the ISU-VDL standard operating procedure to determine surface antigen and associated toxin genes.

Histopathology.

Tissue sections were collected in 10% neutral buffered formalin and allowed to fix for 24h hours. Tissues were then placed in 70% ethanol until routine tissue sectioning followed by paraffin embedding and staining with hematoxylin and eosin. All tissues were examined by a veterinary pathologist (DMM) blinded to the experimental study (DOSAGE, ANTIMICROBIAL, and AGE) and group designation. Large intestinal sections were assessed for goblet cells loss, neutrophilic aggregates within the lamina propria, and mucosal epithelial defects as previously described (Yaeger et al., 2007) with modification. Neutrophilic aggregate scoring was adjusted to include the following: 0 = 0-3 neutrophils, 1 = 3-20 aggregated neutrophils, 20-50 aggregated neutrophils, and 3 = >50 neutrophils. Mucosal epithelial defects were assessed as follows: 0 = normal, 1 = ≤ 4 erosions, 2 = ≥ 5 erosions, and 3 = 1 or more ulcerations. In addition, inflammation within the mesocolon was addressed and scored as follows: 0 = no inflammation, 1 = mild cellular infiltrates, 2 = moderate cellular infiltrates and 3 = marked cellular infiltrates.

Scoring.

Three categories of scores were compared: 1) clinical signs, 2) gross lesions, and 3) microscopic lesions. Clinical signs scores were created by summing scores for body condition, hydration status, and perineum staining. Gross lesion scores were created by summing scores for necrotizing lesions, mesocolonic edema, culture result for C. difficile, and toxin amount. Microscopic lesion score was the sum of scores for all histopathology categories.

Immunohistochemistry

Formalin fixed tissues were placed in 70% ethanol 24 hrs. after collection. Tissues were normally processed into paraffin embedded blocks. Following examination of H & E staining, 30 different pigs within all studies were selected for IHC staining based on microscopic lesions consistent for CdAD. Colonic and/or cecal blocks from selected piglets were cut, deparaffinized, were labeled with four different antibodies directed against Toxin A and Toxin B. A rabbit polyclonal and mouse monoclonal antibody were used for both toxins. Multiple different antigen retrieval methods were applied to all deparaffinized blocks and antibody types and included; heat retrieval in a microwave for 30 minutes,

over-night incubation at room temperature, trypsin, and protease. DAB chromagen was applied for visualization and slides were then counterstained with hematoxylin.

Statistical Analysis.
Scores for clinical signs and gross and microscopic lesions were analyzed by a non parametric test. Wilcoxon/Kruskal test was used to determine if differences existed between comparisons of control groups to isolate groups, and isolate groups to each other. JMP 9 statistical software was used to perform analyses.

Results

Clinical signs.
Clinical scores were independently scored for all pigs within their respective groups and experiments. Results are summarized in Table 2. Statistical evaluation of clinical signs scores from DOSAGE, ANTIMICROBIAL, and AGE experiments revealed no statistical difference ($p>0.05$) among their respective groups.

The majority of pigs at necropsy in the DOSAGE experiment presented with normal body condition as well as hydration status with exception of one animal challenged at $2x10^6$ which presented moderate levels of dehydration. Forty percent (4/10) of piglets challenged at $2 x10^3$ *C. difficile* spores, fifty percent (5/10) of $2x10^6$, and eighty percent (8/10) of $2x10^9$ presented with staining of perineum at necropsy. Three control pigs also had mild staining

In the ANTIMICROBIAL experiment, piglets were mildly to severely dehydrated (37/39) at 72 hours post inoculation and had moderate to marked fecal staining of the perineum (38/39). All but one or two piglets in each group (33/39) were noted to be thin or emaciated.

Piglets from all groups in the AGE experiment were of normal body condition, hydration status, and had no perineal fecal staining 72 hours post inoculation with few exceptions. One piglet in group 10 was thin and two piglets in group 13 had fecal staining of the perineum.

Gross lesions.

Statistical differences were not detected between groups of the DOSAGE, ANTIMICROBIAL, and AGE experiments. Grossly visible mucosal necrosis was not seen within the cecum or spiral colon of in individual piglets in all experiments. Gross lesions by experiment are summarized in table 3.

The gross lesions score for the DOSAGE experiment was not statistically different between groups. However, the score of group 1 was numerically lower than groups 2, 3, and 4. No mesocolonic edema was reported group 1. Mesocolonic edema presented in 30% of group 2 piglets and 40% of piglets in groups 3 and 4.

Within the ANTIMICROBIAL experiment, mesocolonic edema was reported at different frequency among all groups. Edema was observed in one pig each from groups 5, 6, and 8. Two piglets in group 7 and four from group 9 had mesocolonic edema 72 hours post inoculation.

Five piglets in group 10 of the AGE experiment developed mild mesocolonic edema. In group 11, four piglets had edema. Three and five piglets had mesocolonic edema at necropsy in groups 12 and 13, respectively.

Microscopic lesions.

Classical microscopic lesions with high numbers of neutrophils infiltrating the lamina propria, a loss of goblet cells and the presence of single to multiple sites of erosions and ulcerations were observed in sections of colon and cecum from *C. difficile* challenged piglets in the DOSAGE, ANTIMICROBIAL, and AGE experiments. Observed microscopic lesions are for all experiments are detailed in Table 4. Significant changes between groups were not observed in the DOSAGE, ANTIMICROBIAL, and AGE experiments.

For the DOSAGE study, overall microscopic lesions scores were not statistically different between groups (p=0.2), however, there is a clear numeric trend between dose of *C. difficile* and associated microscopic lesions (figure 1). Lesion severity was elevated in animals challenged at higher doses when compared to lower doses and negative piglets. Only rare aggregates were seen within the group 1 piglets. No goblet cell loss or mucosal alterations were seen.

No small intestinal bacterial adherence was observed in any pig from all experiments.

Toxin ELISA

Prior to *C. difficile* inoculation, rectal swabs from all pigs in the DOSAGE ANTIMICROBIAL, and AGE experiments were negative for both toxins A and/or B. Toxin ELISA at termination of experiments is summarized in Table 5.

All the group 1 piglets in the DOSAGE experiment remained toxin negative. Fifty percent of group 2 piglets were toxin positive at necropsy. Moderate to high levels of toxin were detected in four of the five piglets. Similarly, 50% of group 3 piglets were toxin positive 72 hours after inoculation. Toxin quantity varied from low to high in these 5 piglets. Alternatively, 90% of group 4 piglets were toxin positive 72 hours following inoculation. All positive pigs in group 4 had moderate to high levels, 3 or 4+, of toxin present.

Toxin levels were not detected in any group 6 piglet from the ANTIMICROBIAL experiment. Low to moderate levels of toxin were detected in 3/8 (37.5%) group 7 piglets. Two group 8 and one group 9 piglet had low amounts of C. difficile toxin present at 72 hours post inoculation. Within the positive control piglets, group 5, one piglet was toxin positive. However, different than other groups, toxin was detected at high levels (table 3).

In the AGE experiment, no group 10 piglets were positive of C. difficile toxin at necropsy. Toxin was detected in 3/8 (37.5%) group 11 piglets. Two piglets from group 12 and 13 had detectable toxin.

Bacterial Culture.

Clostridium difficile culture results for the DOSAGE, ANTIMICROBIAL, and AGE experiments are summarized in Table 5. No *Salmonella* spp was isolated from any pig. Aerobic and anaerobic small intestinal isolation from all experiments is detailed in Table6.

Twelve C. perfringens isolates were randomly selected for PCR, and all were determined to be type A. Alpha toxin was detected in all isolates. Genes associated with Beta 2 toxin were found in 3 isolates; all others were negative. Twelve hemolytic and twelve non-hemolytic isolates randomly selected were all negative for pillus antigen and associated toxin genes.

Immunohistochemistry

Staining of blocks with either monoclonal or polyclonal antibodies for Toxin A and B was not confirmed. Multiple antigen retrieval methods were used.

Discussion

Several studies have reported that neonatal piglets are susceptible to *C. difficile* toxin which results in yellow pasty diarrhea. [13,23,24] Pre-weaning mortality, poor growth rates and variation in piglet weight at weaning are some of the problems associated with this scenario. [25] Although the awareness of this disease has increased in swine medicine over the last decade, more research is needed to better understand basic principles such as prevention, risk factors, epidemiology and treatment. Currently, there is a lack of a commercial product aiming the prevention of *C. difficile* disease. The treatment of affected animals is occasionally complicated by the fact that this bacterium is resistance to different classes of antibiotics. [26] A better understanding of the risk factors or triggers is very important in order to effectively prevent or minimize the occurrence of the disease. This paper evaluated the association of bacteria dosage with clinical and histological lesions as well as it relates to piglet's age and the possible influence of the use of certain classes of antibiotics on the occurrence and severity of disease.

The procedure of the piglet collection was described previously where animals were collected after normal parturition at the farm. Due to this procedure, some animals may have colonized naturally with endemic microorganisms present in the farm. This was very important for these studies as the dynamic of microorganism succession and competition is believed to be the key factor for disease development. Therefore some of the control piglets were colonized with *C.* difficile, which agrees with the current literature. It was recently reported that piglets started to become colonized within a few hours post-farrow, and within 48 hours of life 100% of farm-raised piglets were colonized [5]. A small percentage of control piglets were naturally colonized by *C. difficile,* however all of them were toxin negative throughout the study period.

Piglets from all three experiments were blindly scored at necropsy at several different parameters which were summarized in clinical and gross lesions score. High percentage of piglets developed diarrhea and were moderate dehydrate at necropsy, however the large normal variation in those variables and the small numbers of piglets per group might have played an important role in the statistical analyses.

It was believed that the *C. difficile* was mainly shed by the sows in farrowing crates and that was the main via of exposure to neonate piglets. However, recent studies were able to demonstrate that only about 25% of the sows shed the organisms at farrowing. [7] Studies investigating the source of *C. difficile* to piglets have concluded that neonate piglets, ambient air samples, and environment are the major source of infection. [5,27] *C. difficile* is a spore forming bacteria and as a survival mechanism for adverse environment the bacteria form spores which are highly resistant to physical and chemical agents such as farm cleaning procedures and most common disinfectants. They can survive for many months in the environment. [28] Histopathological results revealed an interesting trend in the DOSAGE experiment, based on these results it appears that *C. difficile* dose has a direct correlation with microscopic lesions (figure 1).

It is therefore speculated that high shedding sows or poor sanitation within the farrowing crates would lead to disease or individual piglets or litters.

Clostridium difficile disease is the major cause of antibiotic-associated diarrhea in humans [29,30,10,31,12,13] and the same pattern of increased disease incidence is noticed in veterinary medicine, where the majority of cases are associated with disequilibrium of common intestinal flora, due to antibiotic treatment or age of animals.[14] In this study, histological lesions associated with CdAD were observed in piglets enrolled in the ANTIMICROBIAL experiment however not statistical difference was demonstrated among groups. A considerable percentage of piglets were toxin negative and culture negative at the end of the study. It appears that antibiotic treatment do not play an important role in the development and/or in the severity of lesions. However, larger numbers of piglets in a field setting would be needed to confirm this experimental finding. What is not known is whether piglets in this experiment would have developed more severe disease if additional days post-inoculation were monitored. In other species where the antibiotic therapy is important to the development of disease, the main role of it is to disrupt the normal intestinal flora. One-day-old piglets do not have an established intestinal flora and therefore the antibiotic treatment might not have significantly altered the intestinal microflora. This is plausible reason why the antibiotic therapy did not influence the development and severity of disease in the ANTIMICROBIAL experiment. Furthermore, the antibiotic resistance profile of *C. difficile* strains associated to human disease is identified however studies investigating the resistance profile of piglet isolates are lacking. This could potential affect the outcome of CdAD in neonatal piglets if metaphylatic antibiotics are used to mitigate disease pressures after birth.

It is a consensus in the literature that a large majority of cases of CdAD in piglets happens within the first two days of life however in our third experiment we were able to cause disease in 10-days old piglets. Indicating that in the pigs, CdAD is not an age is not a factor of disease. Receptor mediated disease can still occur in this age of piglet. The results of the AGE experiment should be interpreted with consideration since piglets were kept under a controlled environment until they reached 10 days of age. This limited and altered the normal colonization of gut flora which would not normally have occurred in piglets raised in the farm environment. Therefore, it is CdAD in piglets is likely a disease of altered intestinal microflora as in humans and other species. Another factor that differentiates experiment piglets from piglets raised in the farm is the fact that piglets raised with the sow feed several times a day directly from the sow which is a rich source of antibodies, mainly IgA. This would potential impact the normal intestinal flora as well.

In conclusion, the results demonstrate that our isolate is capable of causing microscopic lesions and that C. difficile dose appears to be an important factor that influences the appearance and severity of lesions. In this study, antibiotic administration appeared to not influence the appearance and severity of

lesions. It was also demonstrated that 10 day-old-piglets are also susceptible to the developmental of disease in special circumstances. More studies are needed to better understand risk factors, epidemiology, and the contribution of antibiotic treatment in the prevalence and severity of *C. difficile* disease in piglets.

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Dissemination

This information has been presented at or accepted for presentation at the following.

1. Iowa State University Swine Disease Conference
2. 2012 IPVS, *abstract submitted*

Currently this information is being prepared for journal submission and publication.

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Table1. Experimental design.

Experiment	Groups	*n*	Age	Inoculation & dose‡	Treatment*
DOSAGE	1	10	1 day	None (negative control)	N/A
	2	10		2 x10^3 *C. difficile* spores	
	3	10		2 x 10^6 *C. difficile* spores	
	4	10		2 x 10^9 *C. difficile* spores	
ANTIMICROBIAL	5	8	1 day	2 x 10^6 *C. difficile* spores	None
	6	8		2 x 10^6 *C. difficile* spores	Lincomycin
	7	8		2 x 10^6 *C. difficile* spores	Ceftiofur
	8	8		2 x 10^6 *C. difficile* spores	Tylocin
	9	7		2 x 10^6 *C. difficile* spores	Tulathromycin
AGE	10	7	10 day	None (negative control)	N/A
	11	8		2 x10^3 *C. difficile* spores	
	12	9		2 x 10^6 *C. difficile* spores	
	13	9		2 x 10^9 *C. difficile* spores	

‡ Heat-shocked *C. difficile* spores

* Antibiotic doses were administered per label base on weight and given as directed intramuscularly 16 hours post inoculation

Table 2. Summarized clinical signs by experiment and group. Clinical signs scores included body condition, hydration and perineum fecal staining at 72 hours post inoculation with sham or heat shock *C. Difficile* spores.

Clinical signs								
			Body Condition		Hydration Status		Perineum Staining	
Experiment	Group	*n*	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)
DOSAGE	1	10	0-1	0.2 (0.13)	0-1	0.3(0.15)	0-1	0.3(0.15)
	2	10	0	0 (0)	0-1	0.3(0.15)	0-2	0.7(0.3)
	3	10	0-1	0.2 (0.13)	0-2	0.6(0.22)	0-2	0.7(0.26)
	4	10	0-1	0.2 (0.13)	0-1	0.4(0.16)	0-2	1(0.21)
ANTIMICROBIAL	5	8	0-2	1.1(0.22)	0-3	1.7(0.36)	1-3	2.2(0.36)
	6	8	0-2	1.5(0.32)	0-3	1.8(0.44)	1-3	2.6(0.26)
	7	8	0-2	1.5(0.26)	1-3	2(0.37)	0-3	2.1(0.39)
	8	7	0-2	1.14(0.26)	1-3	2(0.3)	1-3	2.5(0.29)
	9	8	0-2	1.2(0.25)	1-3	2.1(0.64)	2-3	2.6(0.18)
AGE	10	7	0	0(0)	0(0)	0	0	0(0)
	11	8	0	0(0)	0	0(0)	0	0(0)
	12	9	0	0(0)	0	0(0)	0	0(0)
	13	9	0-1	0.1(0.1)	0-1	0.2(0.14)	0-44	0.2(0.29)

12 Table 3. Gross lesion scores including *C. difficile* culture and toxin results by experiment and group.
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Gross Lesions										
			Necrotizing Lesions		Mesocolonic Edema		Culture		Toxin	
Experiment	Group	*n*	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)
DOSAGE	1	10	0	0	0	0(0)	0-2	1.1(0.27)	0	0(0)
	2	10	0	0	0-3	0.5(0.3)	0-3	1.3(0.33)	0-4	1.7(0.59)
	3	10	0	0	0-3	0.8(0.35)	0-3	0.4(0.3)	0-4	1.3(0.49)
	4	10	0	0	0-3	1(0.44)	0-3	2.4(0.3)	0-4	3(0.36)
ANTIMICROBIAL	5	8	0	0	0-3	0.37(0.37)	0-2	1.2(0.25)	0-4	0.5(0.5)
	6	8	0	0	0-2	0.25(0.25)	0-1	0.12(0.12)	0	0(0)
	7	8	0	0	0-3	0.5(0.37)	0-2	1(0.32)	0-2	0.6(0.32)
	8	7	0	0	0-1	0.14(0.14)	0-1	0.5(0.2)	0-1	0.28(0.18)
	9	8	0	0	0-1	0.5(0.18)	0-2	0.3(0.26)	0-1	0.12(0.12)
AGE	10	7	0	0	0-1	0.71(0.18)	0-1	0.5(0.2)	0	0(0)
	11	8	0	0	0-3	0.87(0.39)	0-3	1.8(0.39)	0-2	0.5(0.26)
	12	9	0	0	0-2	0.44(0.24)	0-4	2.3(0.37)	0-3	0.4(0.33)
	13	9	0	0	0-3	0.77(0.32)	0-3	2(0.33)	0-3	0.5(0.37)

Table 4. Summarized microscopic lesions 72 hours post inoculation in large intestine of piglets sham challenged or challenged with heat shocked *C. difficile* spores by experiment and group.

Microscopic Lesions										
			Goblet cell loss		Neutrophilic inflammation		Mucosal defects		Mesenteritis	
Experiment	Group	*n*	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)	Range	Mean (+/- SE)
DOSAGE	1	10	0	0(0)	0-2	0.7(0.26)	0	0(0)	0	0(0)
	2	10	0-3	0.8(0.41)	0-3	0.7(0.33)	0-3	0.4(0.3)	0-1	0.1((0.1)
	3	10	0-3	0.9(0.37)	0-3	1.2(0.41)	0-3	0.9(0.4)	0-2	0.4(0.22)
	4	10	0-3	1.4(0.42)	0-3	1.4(0.42)	0-3	1.2(0.44)	0-2	0.7(0.26)
ANTIMICROBIAL	5	8	0-3	0.75(0.41)	0-3	0.87(0.39)	0-3	0.5(0.37)	0-1	0.25(0.16)
	6	8	0-3	0.5(0.37)	0-3	0.62(0.41)	0-2	0.25(0.25)	0-1	0.12(0.12)
	7	8	0-3	0.37(0.37)	0-3	0.5(0.37)	0-3	0.37(0.37)	0-1	0.12(0.12)
	8	7	0-2	0.28(0.28)	0-2	0.57(0.29)	0-1	0.14(0.14)	0-1	0.14(0.14)
	9	8	0-1	0.25(0.16)	0-2	0.5(0.26)	0-1	0.12(0.12)	0	0(0)
AGE	10	7	0-2	0.71(0.36)	0-3	1.14(0.53)	0-3	0.71(0.47)	0-1	0.14(0.14)
	11	8	0-2	0.25(0.25)	0-3	0.62(0.37)	0-1	0.12(0.12)	0-3	0.5(0.37)
	12	9	0-1	0.11(0.11)	0-1	0.33(0.16)	0	0(0)	0-3	0.77(0.36)
	13	9	0-3	0.88(0.45)	0-3	1.11(0.48)	0-3	0.88(0.45)	0-3	1(0.44)

Table 5. Small Intestine culture results by experiment. At necropsy, luminal swabs from the ileum were cultured.

Experiment	Group	Hemolytic *E. coli**	*C. perf* †	Mucoid/Smooth	Treatment¶
DOSAGE	1	0/10	2/10	0/10	.
	2	0/10	0/10	3/10	.
	3	2/10	0/10	1/10	.
	4	1/10	0/10	2/10	.
ANTIMICROBIAL	5	2/8	1/8	1/8	.
	6	7/8	1/8	1/8	Lincomycin
	7	5/8	1/8	1/8	Ceftiofur
	8	1/7	1/7	1/7	Tylocin
	9	1/8	0/8	1/8	Tulathromycin
AGE	10	3/7	0/7	1/7	.
	11	5/8	0/8	4/8	.
	12	1/8	0/8	1/8	.
	13	4/9	0/9	0/9	.

* Number of samples culture positive for hemolytic *Escherichia coli* per total number of piglets within each group.

† Number of samples culture positive for *Clostridium perfringens* per total number of piglets within each group.

‡ Number of samples culture positive for smooth or mucoid *Escherichia coli* per total number of piglets within each group.

¶ Only piglets enrolled in the ANTIMICROBIAL experiment were treated; 16 hours post inoculation with *C. Difficile*.

Table 6. *Clostridium difficile* culture and toxin ELISA results by experiment. C. difficile culture and toxin ELISA was performed on pooled large intestinal contents and colonic mucosal scrapings.

Experiment	Group	*C. difficile* culture*	Toxin ELISA†	Treatment¶
DOSAGE	1	2/10	0/10	.
	2	7/10	5/10	.
	3	2/10	5/10	.
	4	9/10	9/10	.
ANTIMICROBIAL	5	6/8	7/8	.
	6	0/8	1/8	Lincomycin
	7	3/8	5/8	Ceftiofur
	8	0/7	4/7	Tylocin
	9	1/8	2/8	Tulathromycin
AGE	10	4/7	2/9	.
	11	7/8	1/8	.
	12	8/9	3/8	.
	13	7/9	2/9	.

* Number of samples culture positive for *C. difficile* per total number of piglets within each group.

† Number of samples Toxin positive for *C. difficile* per total number of piglets within each group.

¶ Only piglets enrolled in the ANTIMICROBIAL experiment were treated; 16 hours post inoculation with *C. Difficile*.

Figure 1. Mean microscopic lesions by group for the DOSAGE experiment. Significant differences were not achieved between groups; however there is a clear trend with increased dose of C. difficile and microscopic lesions.