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Pathogenesis and toxins

Effect of age, dose and antibiotic therapy on the development of *Clostridium difficile* infection in neonatal piglets

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ABSTRACT

Piglet diarrhea is associated with increased pre-weaning mortality, poor growth rates, and variation in weight at weaning. *Clostridium difficile* is a known cause of enteric disease in neonatal piglets, yet risk factors associated with *C. difficile* infection in piglets are unknown. The objectives of this study were (1) to evaluate the consistency and severity of lesions in piglets challenged with *C. difficile* at different bacterial doses (DOSAGE experiment), (2) evaluate the use of antibiotics as a contributing risk factor in 1-day-old piglets (ANTIMICROBIAL experiment), and (3) to provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets (AGE experiment). One hundred and eleven conventional neonatal pigs were snatch farrowed and divided into experimental groups addressing the objectives. In the DOSAGE experiment, 40 1-day-old piglets were sham inoculated or challenged with varying amounts of *C. difficile* heat shocked spores and euthanized 72 h post infection. Results indicate a clear trend for disease development as bacterial numbers increase. In the ANTIMICROBIAL experiment, 39 1-day-old piglets were challenged and then treated with one of four different antibiotics after 16 h. No significant difference in disease development was found. Thirty-three 10-day-old piglets were given varying doses of *C. difficile* in the AGE experiment. Disease and lesions were reproduced in 10-day-old piglets. Combined results 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 *C. difficile*, and antibiotic administration following inoculation may not be a major contributor for disease in neonatal piglets.

1. Introduction

Clostridium difficile is a Gram positive, anaerobic, spore-forming bacterium first described as part of the neonatal intestinal flora in 1935 [1]. Subsequently, in 1978, *C. difficile* was linked to human colitis [2], and is now a significant cause of antibiotic-associated diarrhea in several countries worldwide [3–8]. *C. difficile* infection (CDI) in humans is characterized by mild to severe diarrhea, pseudomembranous colitis, and, in the most severe cases, by paralytic ileus, toxic megacolon, bowel perforation, peritonitis, and death. CDI has also been described in several non-human species including pigs, horses, primates, rabbits, rats, domestic dogs and domestic cats [1,9–12]; disease is typically life-threatening only in horses.

The incidence of CDI has been steadily increasing in veterinary medicine. The majority of cases are associated with disequilibrium of commensal intestinal flora. Neonates, as well as animals treated with select antimicrobials, are most commonly affected [13,14], and the hypothesis of causation is that antimicrobials eliminate susceptible microflora within the intestine, thereby allowing strains of *C. difficile* to establish in empty niches and overgrow due to lack of competition.

CDI in piglets is associated with large bowel inflammation, with the potential for pseudomembrane formation. Gnotobiotic pigs have been described as also having systemic disease resulting in ascites, pleural effusion, hepatic abscess, renal dysfunction, and acute respiratory distress [8]. The mechanism by which the microorganism causes systemic disease is not completely understood. Toxin A (TcdA), toxin B (TcdB), and binary toxin (CDT) are known products of many strains of *C. difficile*. TcdA and TcdB are large polypeptides, which are believed to be essential virulence

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factors associated with disease development [1,15]. TcdA is known for enterotoxicity while TcdB is a potent cytotoxin and enterotoxin *in vivo* [5]. There are reports of failed disease associated with TcdB *in vivo* unless prior cellular damage by TcdA has occurred [5,16]. The significance and accuracy of these observations is in question, especially due to the occurrence of human infections with $TcdA^{-}TcdB^{+}$ strains. A mouse study demonstrated that TcdB is several times more toxic than TcdA and *C. difficile* isolates lacking TcdA are still capable of causing severe disease [17]. These results contradict the theory that TcdA action on tissues is an essential prerequisite to cell damage by TcdB. This is further supported by a more recent study using TcdA or TcdB gene knock out *C. difficile* strains. Reported results from this study indicate both are able to cause cellular alterations *in vitro* and *in vivo* disease in hamsters, highlighting the fact that both toxins are independently capable of producing disease [18].

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 h of birth [11]. In contrast to colonization, CDI does not affect all piglets within a litter, but can manifest as mild to severe diarrhea in 1–7 day-old piglets [11,19]. Neonatal piglets are highly susceptible to colonization, 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, associated toxin profile, and animal age. However, there is a lack of knowledge regarding these aforementioned risk factors in swine. The objectives of this study were (1) to evaluate the consistency and severity of lesions in piglets challenged with different bacterial doses, (2) to evaluate the use of antimicrobials as a contributing risk factor in the development of disease, and (3) to provide a clinical and histological evaluation of *C. difficile* infection in 10-day-old piglets.

2. Material and methods

2.1. Animals

One hundred and eleven conventional neonatal pigs were procured from a 2500 head sow farm located in central Iowa. Procedures involving sow preparation, piglet collection and care were as previously described [20]. Once piglets have been collected and properly processed, colostrum collection and administration was performed as described [20]. Pigs were then transported to a BSL-2 animal facility at Iowa State University.

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

2.2. Housing

Piglets were housed in one of six identical raised plastic tubs partitioned into eight individual pens (approximately 0.7 × 0.7 m) with clear solid plastic dividing walls. Piglets housing and daily care were as described previously [20] with slight modifications. 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 h period to effectively eliminate environmental vegetative cells and spores. All challenged pigs were housed in the same room and airspace. Negative control piglets were housed separately.

2.3. Experimental design

Three separate experiments were completed. Table 1 summarizes the design for all three experiments. 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 effect of piglet age in experiment 3 (AGE). In each experiment, pigs were randomly allocated into four (DOSAGE and AGE) or five (ANTIMICROBIAL) groups using several random number iterations in Microsoft Excel®. 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 *C. difficile* spores were inoculated. Group 1 received sham material and groups 2, 3, and 4 received 2×10^3, 2×10^6, 2×10^9 heat shocked *C. difficile* spores, respectively (Table 1). For the ANTIMICROBIAL experiment, all groups were challenged with 2×10^6 heat shocked *C. difficile* spores. Sixteen hours following *C. difficile* challenge, groups 6, 7, 8, and 9 were each administered a different antibiotic (Table 1). The AGE experiment utilized the same protocol for piglet collection. Piglets were kept for 10 days and then challenged with 2×10^3, 2×10^6, or 2×10^9 *C. difficile* spores (Table 1). Piglets were euthanized 72 h after challenge in all experiments.

2.4. 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 A and/or B (4+) were detected by ELISA (C. DIFFICILE TOX A/B II™, Blacksburg, VA) from the clinically affected piglets. Isolate 15454-1 is ribotype 078, toxinotype V, and contains both toxin A and toxin B gene sequences [21]. The isolate was stored at −80 °C until culture preparation.

Procedures involving *C. difficile* isolation, growth harvesting, and titration of spores was accomplished as previously described [20].

2.5. Inoculation

All piglets were inoculated intragastrically using an eight-gauge rubber French catheter as an oral-gastric tube (Sovereign™, Tyco/Healthcare, Mansfield, MA). Inoculation occurred approximately 4 h 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

Table 1
Experimental design.

Experiment	Groups	*n*	Age	Inoculation & dose[a]	Treatment[b]
DOSAGE	1	10	1 day	None (control)	N/A
	2	10		2 × 103 *C. difficile* spores	
	3	10		2 × 106 *C. difficile* spores	
	4	10		2 × 109 *C. difficile* spores	
ANTIMICROBIAL	5	8	1 day	2 × 106 *C. difficile* spores	None
	6	8		2 × 106 *C. difficile* spores	Lincomycin
	7	8		2 × 106 *C. difficile* spores	Ceftiofur
	8	8		2 × 106 *C. difficile* spores	Tylosin
	9	7		2 × 106 *C. difficile* spores	Tulathromycin
AGE	10	7	10	None (control)	N/A
	11	8	days	2 × 103 *C. difficile* spores	
	12	9		2 × 106 *C. difficile* spores	
	13	9		2 × 109 *C. difficile* spores	

[a] Heat shocked *Clostridium difficile* spores.
[b] Antibiotic doses were administered per label based on weight and given as directed intramuscularly 16 h post inoculation.