RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Epidemiology, Toxino- and Geno-typing of *Clostridium difficile* in swine at farm, slaughter and retail - NPB #07-044 revised

Investigator: Wondwossen A. Gebreyes

Institution: The Ohio State University

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Industry Summary

We investigated the role of pigs in carrying important strains of *C. difficile* and the main goal was to determine the prevalence and compare strains of human and porcine origin. Samples were collected from swine farms in Ohio and North Carolina at farrowing, nursery, and finishing stages. The organism was found in 74.5% of farrowing piglets, 0.45% within nursery pigs, and 0% within finishing pig. A low level of multi-drug resistance was found, although most samples were ciprofloxacin resistant. The majority of isolates were found to be toxigenic. While none of the pigs were found to carry the hypervirulent epidemic strain of human health significance (NAP-1), 82.5% (161/195) were found to carry *C. difficile* strains which are Toxinotype V, a strain previously known to cause disease in humans and isolated from various other animal sources. Pulsed Field Gel Electrophoresis (PFGE) DNA fingerprinting show a high level of diversity among isolates of swine origin, with clustering among farms. The results also show a subset of Toxinotype V strains of swine origin with 100% similarity to human isolate. While it is encouraging that *C. difficile* was not found at the finishing stage of production indicating the absence of food safety concern, the occurrence of hypervirulent strains in swine similar to those of human origin could be significant.

Scientific Abstract

Clostridium difficile is a gram-positive spore-forming anaerobic bacillus pathogenic to humans and animals. The role of pork in *C. difficile* dissemination has not been thoroughly investigated. Specific aims of this project are to investigate whether pigs carry hypervirulent strains of *C. difficile* and to compare phenotypic and genotypic attributes of strains of human and porcine origin. Fecal samples (n=251) were collected from swine farms in Ohio (n=3 farms) and North Carolina [NC] (n= 5 farms) at farrowing, nursery, and finishing stages. Bacteriology was done using conventional approaches. Antimicrobial susceptibility was tested using Epsilometric test for ciprofloxacin, erythromycin, metronidazole, vancomycin, tetracycline, and ampicillin. Prevalence of 74.5% within farrowing piglets, 0.45% within nursery pigs, and 0% within finishing pigs were found. Within farrowing pigs, the prevalence was significantly higher in Ohio (88.5%) than in NC (65.6%). Multi-drug resistance was uncommon although most samples, 81.5% (106/130) were resistant to ciprofloxacin with MIC >32 mg/L. Genotypically, the majority of isolates, 82.6% (428/518), were toxin A+B+. Majorityof the isolates (82.5%; 161/195) were found to carry *C. difficile* strains which are Toxinotype V, binary toxin positive and have a 39 bp deletion in the *tcdC*, a down regulator gene for enterotoxin and cytotoxin (*tcdA* and *tcdB* respectively) production. None of ths isolates

were of the epidemic strain of human health significance that shows 18bp deletion in the downregulator, tcdC gene. Twenty-four pigs (12.3%) were also found to carry more than one strain of *C. difficile.* Characterization of human isolates (n=24) showed two strain types similar to those found in pigs: Toxinotype O and nontoxogenic. Pulsed Field Gel Electrophoresis (PFGE) findings show a high level of genotypic diversity among isolates of swine origin, with clustering among different farms. These results also show a group of Toxinotype V strains of swine origin with 100% similarity to CDC NAP7 isolate of human origin, a designated straisn but distinctly different from the epidemic one (NAP-1). While the absence of *C. difficile* at finishing stage of production is encouraging, the occurrence of hypervirulent strains in swine indicates the public health significance of *C. difficile* of porcine origin.

Introduction

Clostridium difficile is a gram positive spore-forming anaerobic bacillus that is pathogenic to both humans and swine. In swine, *C. difficile* is associated with neonatal enteritis and edema of the mesocolon and colonic serosa. In humans, the pathogen is associated with gastrointestinal infections ranging from asymptomatic colonization to severe diarrhea, pseudomembranous colitis, toxic megacolon, intestinal perforation, and death. Historically, the human cases of *Clostridium difficile* associated disease (CDAD) have been considered nosocomial and correlated to old age, long term hospital stays and antimicrobial usage. Recent increases in community-acquired infections as well as increased virulence associated with *C. difficile* infections strongly indicate risk factors, other than the hospital associated factors, may also be significant contributors. Current research has pointed to the possibility of foodborne transmission, although this has not been investigated in detail.

With the changing epidemiology of CDAD, researchers have begun to look into possible sources of community dissemination of this pathogen. Possible sources include soil, water, pets, animals used for food, meat, and vegetables. Although some research has been done to establish the relationship to foodborne transmission, more detailed work needs to be done, especially in the area of swine related transmission. Previous research has found *C. difficile* in retail meat beef and pork products. These publications have focused on meat products as a source of potential interspecies transmission, but are unable to identify the source of the contamination and no conclusive evidence has shown that *C. difficile* contamination of food has led to clinical *Clostridium difficile* infection in humans.

Objectives

1) Investigate the epidemiology and develop baseline data of *C. difficile* in swine at farm and slaughter

2) To determine whether piglets infected with *C. difficile* can remain carriers at nursery and finishing and can also be potential sources of the organism to other pigs in the farm to processing continuum

3) To assess the food safety significance of *C. difficile* and conduct phenotypic and genotypic analyses of *C. difficile* isolated from swine at farm and slaughter are clonal or distinct,

4) To determine if *C. difficile* isolates from swine production and processing are phenotypically and genotypically similar to the highly virulent strain reported in humans and also other strains identified in veterinary settings previously.

Materials & Methods

Fecal samples from swine have been collected from farms in North Carolina (n=5) and Ohio (n=3). The samples were collected at three stages, beginning with farrowing (1-7 days of age). Positive individuals identified at farrowing were followed and subsequently sampled at nursery (about six weeks of age) and at the finishing farm (26 weeks of age, 24-28 hours before slaughter). Standard methods were used to isolate and identify the bacteria as described previously by Wilson, Kennedy, and Fekety in1982. Three colonies were saved from each positive stool sample. Also, 24 human isolates were collected from a hospital in Columbus, OH. These isolates were previously obtained from clinical cases.

All *C. difficile* isolates were phenotypically characterized by testing them against a panel of antimicrobials including: ciprofloxacin, erythromycin, metronidazole, vancomycin, tetracycline, and ampicillin using the Epsilometric test (E-test). Genotypic analysis was done using PCR based toxinotypes, toxins of the PaLoc region (*tcdA, tcdB* and *tcdC*) and also binary toxin (*cdtB*), PCR-ribotypes and PFGE comparison. Methods used, primers, and interpretation of these tests were done using previously described methods. Reference strains of *C. difficile* of human origin were used for PFGE comparison and were supplied by the United States Centers for Disease Control and Prevention (CDC). PFGE was completed on a total of 90 isolates, including 67 isolates of swine origin, 20 isolates from humans, and 3 CDC isolates, including NAP7, NAP5, and Toxinotype XIV (unnamed) strains.

Results by Objective

1) Investigate the epidemiology and develop baseline data of *C. difficile* in swine at farm and slaughter

Clostridium difficile was isolated from 65.8% of farrowing piglets in North Carolina and 88.5% of farrowing piglets in Ohio (Table 1). At the nursery stage, 1/213 (0.47%) of these pigs, none were found to be positive for *C. difficile* (0/94) at market age (as per fecals collected at slaughter). Multidrug resistance was low with 10.2% (19/187) being resistant to three or more classes of antimicrobials, with the most common pattern being CipEryTet, 94.7% (18/19). One isolate was also found to have a CipEryAmp resistance pattern. The most common antimicrobial resistance pattern found was CipTet, which was found in 32.1% (60/187) of isolates tested. Other common patterns included CipEry in 8.55% (16/187) and EryTet in 5.88% (11/187) of the isolates. The majority of isolates were found to be resistant to Ciprofloxacin (80.5%), while some isolates were found to be resistant to tetracycline (21.5%) and few were found to be resistant to erythromycin (7.48%). Antimicrobial resistance genes were identified using PCR. Resistance to tetracycline was found to be encoded by *tetM* in 100% (84/84) of swine isolates and by *tetW* in 36.9% (31/84). All strains positive for *tetW* were also positive for *tetM*. Resistance to erythromycin was found to be encoded by *ermB* in 82.9% (34/41) of swine isolates phenotypically showing resistance to erythromycin.

PCR toxin profiling showed that 82.9% (428/516) of the isolates had a toxin A+B+ profile, 15.7% (81/516) were toxin A-B- (non-toxigenic), and 1.36% (7/516) were A-B+. On an individual pig level, PCR-RFLP toxinotyping showed that 70.30% (137/195) of the pigs carried only Toxinotype V, 5.13% (10/295) carried only Toxinotype O, 4.62% (9/195) carried both Toxinotype V and O, 12.3% (24/195) carried both Toxinotype V and nontoxogenic, and 7.69% (15/195) carried only nontoxogenic strains of *C. difficile*. Overall, 82.5% (161/195) of sampled pigs carried Toxinotype V and 12.3% (24/195) carried more than one strain. All Toxinotype V strains' toxin were A+B+, the binary toxin (*cdtB*) positive, and had a 39bp deletion in the t*cdC* gene. All Toxinotype O isolates were found to be toxin A+B+, binary toxin negative, and did not have a deletion in the *tcdC* gene.

Farms at farrowing	Prevalence
North Carolina (5 farms)	65.8% (102/155)
Ohio (3 farms)	88.5% (85/96)
Antimicrobial Resistance Patterns (n=187 isolates)	
CipTet	32.1% (60/187)
CipEryTet	9.63% (18/187)
CipEry	8.55% (16/187)
EryTet	5.88% (11/187)
PCR-RFLP Toxinotypes (n=195 pigs)	
Toxinotype V	70.3% (137/195)
Toxinotype V and nontoxogenic	12.3% (24/195)
Nontoxogenic	7.69% (15/195)
Toxinotype O	5.13% (10/195)
Toxinotype V and O	4.62% (9/195)

Table 1: Characterization of *C. difficile* of swine origin

2) To determine whether piglets infected with *C. difficile* can remain carriers at nursery and finishing and can also be potential sources of the organism to other pigs in the farm to processing continuum

Based on the fecal samples collected, it appears as though piglets are not remaining carriers through the growing process (Tables 2-4). Although this is a positive finding, it is also possible that the pigs did continue to carry the organisms at low levels and the detection method used was not sensitive enough to detect it.

FARROWING			
Farm Stage	Pigs Sampled	Positive	Percent Prevalence
North Carolina-1	30	18	60
North Carolina-2	29	17	58.6
North Carolina-3	32	21	65.6
North Carolina-4	32	28	87.5
North Carolina-5	32	18	56.3
Total	**155**	**102**	**65.8**
Ohio-1	32	28	87.5
Ohio-2	32	28	87.5
Ohio-3	32	29	90.6
TOTAL	**96**	**85**	**88.5**

Table 2. Prevalence of *C. difficile* in farrowing piglets

NURSERY			
Farm Stage	**Pigs Sampled**	**Positive**	**Percent Prevalence**
TOTAL	**213**	**1**	**0.47**

Table 3. Prevalence of *C. difficile* in nursery pigs

Slaughter Fecals			
Farm Stage	**Pigs Sampled**	**Positive**	**Percent Prevalence**
TOTAL	**94**	**0**	**0**

Table 4. Prevalence of *C. difficile* in slaughter pigs

3) To assess the food safety significance of *C. difficile* and conduct phenotypic and genotypic analyses of *C. difficile* isolated from swine at farm and slaughter are clonal or distinct,

Based on PCR toxin profiling and PCR-RFLP Toxinotyping, the *C. difficile* isolates from swine appear highly similar to each other implying clonal dissemination. PCR toxin profiling showed that 82.9% (428/516) of the isolates had a toxin A+B+ profile and 82.5% (161/195) of sampled pigs carried Toxinotype V. All Toxinotype V strain's were toxin A+B+, the binary toxin (*cdtB*) positive, and had a 39bp deletion in the t*cdC* gene.

PFGE results showed large diversity among isolates of swine and human origin (Table 5). Three groups of interest were noted specifically in the PFGE dendrogram analysis. All groups are composed of isolates with at least 70% similarity. Group 1 is made up of six isolates that were found to be 100% identical. Five of the isolates from this group are swine isolates from one farm in North Carolina that are all Toxinotype V and the sixth isolate is a human CDC NAP7 Toxinotype V strain. Group 2 is a large group of Toxinotype V isolates, composed of 24 swine isolates from different farms in Ohio and North Carolina. Group 3 is a small group of isolates, containing seven isolates from one Ohio farm and one isolate from a second Ohio farm. In general, the isolates of human origin were found to clustered together based on toxinotype.

PFGE Results				
Cluster type	**# of Isolates**	**Source of isolates**	**Toxinotype**	**% Similarity**
I	6	5 swine(1 NC farm), 1 human (CDC isolate)	V	100%
II	24	swine (NC and OH)	V	83%
III	7	swine (2 different OH farms)	O	100%

Table 5. Summary of PFGE results of *C. difficile* isolates of swine and human origin

4.) To determine if *C. difficile* isolates from swine production and processing are phenotypically and genotypically similar to the highly virulent strain reported in humans and also other strains identified in veterinary settings previously

PCR- RFLP toxinotyping showed that 82.5% of the pigs carried Toxinotype V, a hypervirulent toxinotype previously reported in humans. Most of the isolates of swine origin (82.9%) were found to be Toxin A+B+ CDT+ and carrying a 39 bp deletion in the down regulator (*tcdC)* gene. PFGE results showed a high level of genotypic diversity among the strains of swine origin. PFGE results showing 100% similarity in pulsotype between a group of swine strains and CDC NAP7 Human isolate (Group 1 previously noted). However, none of the strains typed in this study showed the occurrence of the currently epidemic human strain, NAP-1.

Discussion:

C. difficile was found commonly (74.5% prevalence) in farrowing piglets, and the prevalence was relatively low (0.46%) in nursery pigs. Low levels of antimicrobial resistance have been found, though the majority (80.5%) have an MIC >32 for ciprofloxacin. *tetM* was the most common tetracycline resistance gene found in both the swine (100%) and human (50%) isolates. *ermB* was also commonly found in the swine isolates (82.9%) and in the non toxigenic human strains. PCR-RFLP toxinotyping showed that 82.5% of the pigs carried Toxinotype V, a hypervirulent toxinotype previously reported in humans. Most of the isolates of swine origin (82.9%) were found to be Toxin A+B+ CDT+ and carrying a 39 bp deletion in the down regulator (*tcdC)* gene. PFGE results showed a high level of genotypic diversity among the strains of swine origin.

None of the isolates detected in this study were of the epidemic strain of human health significance (NAP-1). In addition, the absence of *C. difficile* at finishing stage of production is encouraging from food safety standpoint. The occurrence of hypervirulent strains previously detected from human patients and other host animal species may indicate the public health significance of *C. difficile* of porcine origin. Concern is also significant with the PFGE results showing 100% similarity between a group of swine strains and CDC NAP7 Human isolate (Group 1 previously noted). Further characterization of strains and comparison to more diverse human isolates from the same geographic location is currently in progress.