COMPARISON OF THE PREVALENCE AND GENOTYPIC CHARACTERISTICS OF *CLOSTRIDIUM DIFFICILE* IN A CLOSED AND INTEGRATED HUMAN AND SWINE POPULATION IN TEXAS

A Dissertation

by

KERI NOELLE NORMAN

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2010

Major Subject: Biomedical Sciences

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Approved by:

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ABSTRACT

Comparison of the Prevalence and Genotypic Characteristics of *Clostridium difficile* in a Closed and Integrated Human and Swine Population in Texas. (August 2010)

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Co-Chairs of Advisory Committee: Dr. H. Morgan Scott
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Clostridium difficile has been recognized as one of the leading causes of nosocomial diarrhea and pseudomembranous colitis in human hospitals and nursing homes since the 1970s; however, recent occurrences of community-acquired cases have led researchers to search for additional sources of these infections. Some of the possible sources being investigated include food animals and retail meat. The objective of this study was to compare the prevalence and genotypic characteristics of *C. difficile* isolated from a closed population in Texas consisting of both humans and swine. Implicit in this objective, we seek to investigate the possible food safety and occupational risks associated with swine and *C. difficile*.

Isolation of *C. difficile* was performed utilizing an enrichment technique and restrictive media. Polymerase chain reaction (PCR) was used to test for the presence of the toxin A and B genes, the *tcdC* gene deletion, and the binary toxin gene. Genotypic characteristics were compared using PCR toxinotyping and pulsed-field gel electrophoresis (PFGE). Antimicrobial susceptibility was tested using commercially available tests (ETest®) for 11 different antibiotics. Statistical comparisons (both

parametric and non-parametric, and appropriate to the data) were performed both between and among host species.

We tested 2,292 aggregated human wastewater samples and 2,936 swine fecal samples from 2004 to 2006 and found 271 (11.8%) and 252 (8.6%) to be positive for *C. difficile*, respectively. The prevalence of *C. difficile* among swine production groups differed significantly ($p<0.05$); however, prevalence in the human occupational group cohorts (swine workers and non-workers) did not differ ($p=0.81$). The majority of the human and swine isolates were a PFGE NAP7 (a variant pattern with 90.5% similarity) toxinotype V strain. Antimicrobial resistance levels and multi-resistance patterns were generally similar between host species; however, there was decreased susceptibility ($p<0.05$) to ampicillin, clindamycin, and imipenem observed in swine isolates, whereas there was decreased susceptibility ($p<0.05$) to ciprofloxacin in the human isolates.

The similarity in *C. difficile* prevalence between swine workers and non-workers suggests a low occupational hazard of working with swine as it relates to *C. difficile* source. We also found that there is a decreased prevalence of *C. difficile* in late production groups in swine suggesting a lowered risk of food-borne exposure. However, the majority of the isolates derived from the human wastewater and swine appeared to be of very similar strain types, suggesting that a common environmental point source predominates for both hosts.

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NOMENCLATURE

bp	base pair
CA-CDAD	Community-acquired *Clostridium difficile*-associated disease
CA-CDI	Community-acquired *Clostridium difficile* infection
CCFA	Cycloserine cefoxitin fructose agar
CCFB	Cycloserine cefoxitin fructose broth
CDAD	*Clostridium difficile*-associated disease
CDMN	*Clostridium difficile* moxalactam norloxacin
CDMNA	*Clostridium difficile* moxalactam norfloxacin agar
MIC	Minimum inhibitory concentration
MLVA	Multilocus variable-number tandem-repeat analysis
NAP	North American pulsed-field type
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
PYG	Peptone yeast glucose
RO	Reverse osmosis
TCDMNB	Sodium taurocholate *Clostridium difficile* moxalactam norfloxacin broth
TCCFB	Sodium taurocholate cycloserine cefoxitin fructose broth
VL	Viande-Levure broth

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1. INTRODUCTION

1.1. Background

Clostridium difficile is one of the most common causes of nosocomial, or hospital-acquired infections (1). *Clostridium difficile* primarily affects individuals with an altered gut flora; this occurs predominately through antibiotic use. This anaerobic, Gram-positive bacterium produces spores that are both heat- and alcohol-resistant and is, therefore, easily transferred between patients and equipment in hospitals. *Clostridium difficile* has been found to colonize approximately 20% of hospitalized patients versus 3% of healthy adults (2). *Clostridium difficile* was originally discovered in the gut flora of infants; however, since infants lack the toxin binding receptors in the colon for *C. difficile*, it does not readily cause disease in that age group (3, 4). As an infant matures, *C. difficile* is replaced by other bacteria (commensals) found in the normal gut flora of healthy adults.

1.1.1 Description

Vegetative *C. difficile* cells are shed along with spores in the feces of patients infected with the bacterium. It has been shown that vegetative cells survive in an aerobic environment for 15 minutes, whereas, spores can survive for a minimum of 24 hours and often survive for much longer periods of time. Vegetative cells grown on nutrient-rich agar can survive under aerobic conditions for approximately 3 hours (5). *Clostridium difficile* agar-cultured colonies are large, flat, and have a broken-glass

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This dissertation follows the style of Emerging Infectious Diseases.

appearance. Gram staining reveals straight rods with subterminal spores ranging in length from 3 to 7 µm and 0.5 to 2 µm in width. One of the few specific biochemical tests for *C. difficile* is the production of the enzyme L-proline-aminopeptidase. The bacterium is typically grown on selective media containing cycloserine, cefoxitin, fructose, and neutral red (6). *Clostridium difficile* is resistant to cycloserine and cefoxitin at concentrations of 250 mg/L and 8 mg/L, respectively (7). *Clostridium difficile* ferments fructose and when used in conjunction with neutral red as a pH indicator the colonies have a yellow appearance on the plates (6).

Clostridium difficile contains two large proteins, toxin A and toxin B, that are the major pathogenicity factors. Toxin A is described as a weak cytotoxin and an enterotoxin and toxin B is a cytotoxin. A cytotoxin has toxic or destructive effects on certain cells and the toxins in *C. difficile* work on several different types of cells. Enterotoxins are protein toxins released in the gastrointestinal tract that cause vomiting, diarrhea, and abdominal pain (8). The genes that encode for these proteins (*tcdA* and *tcdB*) are closely linked and transcribed in a similar manner (9). The negative regulation of the toxin genes is controlled by the *tcdC* gene. This gene prohibits the production of toxin genes until the cell is in the log growth phase (10). There have been several common deletions found in the *tcdC* gene of some strains of *C. difficile*. Some of the common deletions include an 18-bp deletion found in toxinotype III strains and a 39-bp deletion found in toxinotype V isolates. Several researchers have found that deletions in the *tcdC* gene caused strains to become more virulent due to increased toxin production (11). Pathogenic strains of *C. difficile* also contain a binary toxin gene (*cdt*). The roles

and importance of this gene in the pathogenicity of *C. difficile* are not yet known (12). The pathogenicity locus (PaLoc), where these genes are located, also contains *tcdD* and *tcdE* genes. The roles and importance of these genes are still under investigation (10).

1.1.2 History

Clostridium difficile was first isolated from the stools of infants by Ivan C. Hall and Elizabeth O'Toole in 1935 and was found to be part of the normal gut flora of infants. They called the bacterium *Bacillus difficilis*, because it grew slowly in culture and was difficult to isolate (4). Isolation of *Clostridium difficile* from humans was not reported again until 1943. From 1943 to 1961 there were eight reported isolations of *C. difficile* from hospitalized patients. The bacterium was isolated from a variety of sources and patients with varying demographic characteristics. In 1943, *C. difficile* was found in the muscle tissue of a soldier who died from gas gangrene; in 1957 from an abscessed fracture in an adult male; in 1957 from the blood of a male infant; in 1959 from the pleural fluid of an elderly male; in 1959 from peritoneal fluid of an adolescent girl; in 1959 from pleural fluid of an elderly male; in 1959 from an elderly woman with fatal peritonitis; and in 1961 from an abscess in an adult woman (13).

In 1977, *C. difficile* was found to be the cause of antibiotic-associated pseudomembranous colitis (14). Pseudomembranous colitis is manifested as severe inflammation with copious inflammatory byproducts in the colon that are produced by the disruption of the normal gut flora following the administration of antibiotics (15). Since its discovery as the cause of pseudomembranous colitis, *C. difficile* has been identified as a common problem in hospitals and nursing homes.

1.1.3 Taxonomy

The association between *C. difficile* and pseudomembranous colitis, antibiotic-associated diarrhea, and inflammatory bowel disease led researchers to study more carefully *C. difficile* throughout the late 1970's and 1980's. In the early 1980's, scientists began to experiment with different typing schemes to differentiate between strains of *C. difficile*. The first typing scheme involved bacteriophages (16-18). Another typing method that was used in the late 1980's was sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) of sulphur-35-methionine-labeled proteins (19, 20). Researchers found the method to be simple, rapid, and reproducible; however, there were strains that they were not able to type (19).

In 1987, a correlation was found between groups of *C. difficile* typed by SDS-PAGE and toxin production. Researchers found that isolates that did not produce toxin were in a different group from those strains that produced toxin (21). At the same time, a study was published comparing restriction endonuclease DNA analysis (REA) with SDS-PAGE as a typing method (20). This method was used to type isolates that previously could not be typed using bacteriophage or SDS-PAGE and the researchers found that the *HindIII* restriction enzyme was the most suitable for REA (22). The first paper describing a ribotyping technique was published in 1993 (21). This typing method used polymerase chain reaction (PCR) to look at variable lengths in the 16S and 23S rRNA intergenic spacer regions. This method was used to type more isolates than the bacteriophage or SDS-PAGE methods and the results were easier to read than REA, because fewer bands were produced (23). By the mid-1990's, pulsed-field gel

electrophoresis (PFGE) was also used and compared to other typing methods for *C. difficile*. PFGE uses restriction enzymes to cut DNA into large fragments that are then run through a gel where the voltage is periodically switched between three directions (24-26). In 1998, a new typing method was introduced that looked at the differences in the genes coding for the toxin A and toxin B genes. In this method, referred to as toxinotyping, PCR was used to amplify the toxin genes and then restriction enzymes were used to observe restriction fragment length polymorphisms (RFLP's) (27).

Currently, the three most widely reported typing methods are toxinotyping, ribotyping, and PFGE. PFGE is more commonly used in the United States, whereas ribotyping is more commonly used in Europe (28). Toxinotyping is a relatively easy way to subtype isolates, because it uses well-published and standardized PCR and REA techniques (27). Several different toxinotypes can fall into the same PFGE or ribotype group; however, the number of PFGE and ribotyping patterns found exceeds the number of toxinotypes. Isolates in the same toxinotype have many identical characteristics such as presence or absence of the toxin genes, presence or absence of the binary toxin gene, and size of the *tcdC* gene deletion, and yet this often provides little information regarding the relatedness of the strains (29). PFGE and ribotyping produce visual banding results that are more readily comparable using dendrograms than toxinotyping, but also have their disadvantages. PFGE is requires expensive equipment and is time consuming; however, it produces banding patterns that are easily interpreted. Ribotyping does not require expensive equipment; however, banding patterns are more difficult to interpret (30). There are no worldwide standardized typing protocols for *C.*

difficile and new typing methods, such as multilocus variable-number tandem-repeat analysis (MLVA) and multilocus sequence typing (MLST), are still in early stages of investigation (31). MLVA exhibits a high level of discrimination; however, some researchers have ironically found it to be too discriminatory with similarities between strains that were seen with PFGE no longer apparent in a MLVA dendrogram (29). MLST is more labor intensive than the other techniques, because it sequences multiple loci and a limited number of researchers have access to sequencing equipment; however, the data produced is consistent between laboratories and can reveal genetic lineage between strains (29, 32). The field molecular microbiology is very "fluid" and likely to change in the near future as costs decrease exponentially, making it important to report and compare as many typing methods as is practicable, along with conserving isolated strains for future analysis.

1.1.4 Clinical significance

Clostridium difficile is a major problem in hospitals and nursing homes and costs the United States health care system nearly $1.1 billion per year. Patients infected with *C. difficile* experience symptoms ranging from diarrhea to severe cases of colitis that may result in death (33). Patients testing positive for presence of *C. difficile* in stool samples and also experiencing symptoms such as diarrhea are presumptively diagnosed as having *C. difficile*-associated disease (CDAD). Treatment of CDAD is difficult because of its direct causal relationship with antibiotic use. Initial treatment of *C. difficile* usually involves immediate discontinuation of antibiotic treatment followed by fluid replacement. If this treatment is ineffective, the most common antibiotics

prescribed are metronidazole and vancomycin. However, resistance of *C. difficile* to metronidazole and vancomycin sometimes leads to treatment failure or relapses of infection (34). The risk of *C. difficile* infection is increased if the bacteria are resistant to antimicrobials drugs (35).

1.1.5 Study significance

Recently, attention has been given to a new strain of *C. difficile* (PFGE type NAP1, REA group B1, ribotype 027, toxinotype III) that is believed to be more virulent than previously cultured strains (36, 37). Outbreaks of this hyper-virulent strain have been seen both in North America and Europe (36, 38). This strain contains an 18-bp deletion in the *tcdC* gene, which is a negative regulatory gene for the production of toxins A and B (11, 39, 40). It is believed that a deletion in this gene leads to increased toxin production, because of decreased inhibition of toxin production (41). This strain also contains a binary toxin gene that was rarely found in previously reported strains (42). The role of the binary toxin in the pathogenicity of the bacteria is not yet known; however, it is believed to be partially responsible for the increased virulence of toxinotype III isolates (43, 44).

The emergence of community-acquired *C. difficile* infections in the last few years has completely changed the previous perception that *C. difficile* was primarily a hospital or antibiotic-treatment related acquired infection. Community-acquired cases are classified as patients with no history of hospitalization and many of them also have no history of antibiotic use (45). In the past, patients infected with *C. difficile* were easily identified as having one or several well known risk factors, including

hospitalization, antibiotic use, and were often elderly individuals. The risk factors for and source of community-acquired infections is not known; however, there are several hypotheses that are currently being investigated (46). Some of the hypotheses being investigated include food-borne exposure, companion and food animal exposure, environmental exposure, use of proton pump inhibitors, and microbiological laboratory exposure (47-60).

Clostridium difficile has been isolated from food animals including swine, chicken, and cattle. Some strains isolated from swine have shown as much as an 80% similarity to those isolated from humans (54). *Clostridium difficile* has also been isolated from retail meat (48, 56). The finding of *C. difficile* in food animals and retail meat raises concern for the potential for both food-borne and occupational exposures.

1.2 Study objectives

The objectives of this study were to compare the prevalence and genotypic characteristics of *C. difficile* isolated from closed and integrated human and swine populations in Texas from 2004-2006. We compared the prevalence of *C. difficile* in the various swine production groups to explore the potential for food-borne exposure in market-aged pigs. The prevalence in a swine worker cohort was compared to a non-worker cohort to explore possible occupational exposure. The isolates from the swine and human samples in this closed population were compared to determine genotypic similarity of isolate carriage among the two host species. Finally, we compared both within and between the host species the antimicrobial susceptibility of isolates arising from this closed population

2. LITERATURE REVIEW

2.1 Background

Clostridium difficile is one of the most common causes of nosocomial diarrhea (61). Many studies have been conducted looking at the risk factors for *C. difficile*-associated disease (CDAD) in hospitals and nursing homes (62-66) and ways to both prevent (67-73) and treat the disease (74-80). Recently, cases of CDAD have been found in populations previously considered to be low-risk, and the focus of *C. difficile* studies has shifted to understanding community-acquired cases of CDAD (36, 44, 81-82). Community-acquired CDAD (CA-CDAD) has been discussed in the literature since the 1990's (81); however, the studies themselves were not designed to investigate the fraction of community-acquired cases among the overall total (including nosocomials), and it is arguable as to whether or not these cases are truly community-acquired.

As these community-acquired cases began to be recognized, studies were conducted to evaluate risk factors associated with these cases. Factors including antibiotic use, age, and underlying conditions that are typically associated with hospital-acquired CDAD were also found to be associated with CA-CDAD (82-84). However, there remained cases of CA-CDAD that were not associated with any of these common risk factors (85). Recently, the two most commonly investigated risks for CA-CDAD have been the use of gastric acid suppressants (86) and exposure to food animals and their products (51-54, 58, 87, 88).

Researchers hypothesize that changes in the digestive tract from gastric acid suppressants may facilitate the colonization of *C. difficile* or promote the survival of

spores (89). There have been studies both supporting (59, 90-92) and refuting (93, 94) gastric acid suppressants as a risk factor for hospital-acquired CDAD and this is a hypothesis currently being heavily investigated for CA-CDAD.

Clostridium difficile has been isolated from a variety of food animals including swine, cattle, and chickens (51-54, 58, 87, 88). *Clostridium difficile* is suspected to cause diarrhea in calves (95) and is a known cause of pseudomembranous colitis and enteritis in piglets (87, 96-99). The finding of *C. difficile* in food animals has led scientists to speculate that they may be a source of CA-CDAD. Studies evaluating the transmission of the bacterium between animals and humans have focused on the molecular similarity between strains (53, 54, 100-102), and one study found 100% similarity between swine and human strains (54). Further findings of *C. difficile* in retail meat intended for human consumption has led to more research and continued studies in this area (48, 50, 103).

There have been limited studies investigating the significance of asymptomatic human carriers of *C. difficile (104, 105)*. The majority of investigations have been conducted in health care facilities and do not typically address the concerns of community-acquired cases. Asymptomatic carriers may serve as an important source and route of transmission for CA-CDAD. Conducting studies on CA-CDAD in the population-at-large is difficult and human wastewater samples may provide a valuable source to obtain information enteric bacteria arising from human populations outside health care facilities. *Clostridium difficile* has been detected in biosolids from

wastewater facilities (106); however, the relevance of these findings to human populations is unknown.

2.2 Community-acquired Clostridium difficile

Community-acquired *C. difficile* infections have recently gained attention, because of their occurrence in low-risk populations and an increase in the number of CDAD patients with no previous history of hospitalization (45). Studies conducted since the 1990's have compared community-acquired CDAD to hospital-acquired infection (37, 44, 81, 107-109); however, until recently there have been few reported studies that have explored the risk factors associated with community-acquired infections. The most common risk factor for CA-CDAD is previous antibiotic use (82-84); however, there are many cases of community-acquired CDAD with no history of antibiotic use. Gastric acid suppressants are one of many other risk factors being explored for community-acquired CDAD (86).

2.2.1 Community- vs. hospital-acquired Clostridium difficile

Community-acquired (CA) *C. difficile* was first mentioned in 1990 in a prospective case-control study examining the incidence of *C. difficile* in hospitalized children (81). The researchers compared the prevalence of *C. difficile* in children with gastrointestinal symptoms or who had received antibiotics (the cases; n=337) to children without gastrointestinal symptoms or who had not received antibiotics (the controls; n=57). The authors concluded that the majority of the *C. difficile* infections were, in fact, community-acquired, rather than hospital-acquired, since infection was typically detected within the first 48 hours of hospitalization. The authors failed to mention

whether the patients' previous history of hospitalization was recorded. They found that 96% of the patients with either (or both) gastrointestinal symptoms or previous antibiotic treatment had culturable *C. difficile* in their stools. On the other hand, 5% of the control patients (i.e., with no gastrointestinal symptoms or previous antibiotic treatment) had *C. difficile* in their stools. When only comparing patients previously treated with antibiotics to patients without antibiotic treatment (patients with and without gastrointestinal symptoms were omitted), no difference in *C. difficile* infection rate was found. One of the problems with this report is a lack of data about the age of the children in the study, which could confound the interpretation of results. *Clostridium difficile* has been found to be a part of the gut flora of healthy infants. All of the patients with no gastrointestinal symptoms or previous antibiotic use who had a positive stool sample for *C. difficile* were 0 to 2 months in age (81). The authors failed to provide the age distributions among the cases and controls to eliminate the possibility that there is an age-related confounding effect. The authors themselves even suggest that there would be an increased risk of *C. difficile* infection in children 6 to 12 months in age, because this is when they begin to attend day care. The conclusion that the *C. difficile* infections in this study were community-acquired appears to be premature given that no data on the history of previous hospitalization was provided and the data may need to be adjusted for age. This is one of the first articles to recognize and investigate community-acquired *C. difficile* among patients. It is also interesting to note that there were cases of *C. difficile* with no history of antibiotic use; however, it is not known whether these cases were truly community or hospital-acquired.

In 2006, another study was conducted concerning the epidemiology of *C. difficile* in pediatric patients in California, once again identifying cases of community-acquired *C. difficile* (107). This study collected data on pediatric patients in two different hospitals (one freestanding pediatric hospital and one hospital serving all ages) and tested stool samples for the presence of *C. difficile*. Using the data collected from the patients, the authors identified several risk factors for *C. difficile* colonization in children, and tested the significance of these factors using univariate and multivariable logistic regression. The authors concluded that the majority of their *C. difficile* isolates were community-acquired, because they had unique arbitrarily primed PCR fingerprints; however, there were some similar fingerprints, that may be evidence of nosocomial transmission. The authors found that prior hospitalization, prior infection with *C. difficile*, use of 2 or more antibiotics, and underlying conditions (i.e., any medical condition requiring frequent and long-term medical intervention) were associated with risk of a positive stool sample. We do not know if the isolates with unique fingerprints were from patients with these risk factors or not. A positive stool sample in conjunction with any of these risk factors could be classified as a nosocomial infection, regardless of whether or not it had a unique PCR fingerprint. A unique PCR fingerprint alone is not evidence that an isolate is community-acquired (especially since the organism is so prevalent in the environment). *Clostridium difficile* spores can survive in the environment for long periods of time and patients may not become clinically ill with *C. difficile* until several weeks after they have been released from the hospital. Also, as in the previously cited article, other pathogens including *E. coli*, *Salmonella*, and *Shigella*

were identified in the stool samples along with *C. difficile*. Little is known about how *C. difficile* interacts with other bacteria in the gut, and it is possible that different strains of *C. difficile* are more commonly found in association with other bacteria. It is difficult to conclude from the evidence presented in this article whether or not these truly were community-acquired cases of *C. difficile*.

The epidemiology of CDAD was observed from 2000 to 2004 in a retrospective study published in 2007 (37). A case of CDAD was considered community-acquired if diarrhea occurred in the patient within 72 hours of admission to the hospital or if the patient had not been hospitalized within the past month. During the study, 2,257 patients had *C. difficile* fecal culture performed as directed by a physician, and, of these, 151 met the case definition of CDAD (diarrhea in a hospitalized patient with either (or, both) a positive stool cytotoxicity assay or else toxigenic culture for *C. difficile*). The majority of the patients with CDAD were classified as hospital-acquired (81%); however, 19% of them were classified as community-acquired. One of the interesting findings in this study is that only 10% of the cases that were considered hospital-acquired were infected with strains similar to those found in other patients hospitalized around the same time period. The previously mentioned article by Rexach et. al. (107) classified cases as community- or hospital-acquired based purely on DNA fingerprints of the strain, but this study suggests that patients with characteristics of hospital-acquired infections did not necessarily have similar DNA fingerprints. The authors of this study concluded that only 10% of the hospital-acquired infections were the result of other symptomatic patients and that the other 90% of hospital-acquired infections were a result of

asymptomatic carriers in the hospital. They also found that the strains responsible for hospital-acquired infections were similar to the strains responsible for community-acquired cases of CDAD.

A retrospective case-control study investigating the role of binary toxin positive isolates in CDAD found an association between binary toxin isolates and community-acquired cases (44). Cases were defined as patients with antibiotic-associated diarrhea (AAD) due to a binary toxin positive *C. difficile* isolate. Cases were matched to two controls on the basis of hospital ward and date of hospitalization. Controls were sampled from patients with AAD caused by a binary toxin negative *C. difficile* isolate. Univariate analysis found that cases with binary toxin positive isolates were more often associated with community-acquired infections. One of the problems with this study was that the authors did not define community-acquired infection, which makes it difficult to compare their results to other studies. Another concern with this study is the small sample size. There were 26 cases and 42 controls, and they were only able to match two controls to 16 of the cases; the remaining 10 cases were matched to a single control. This shows that they had a limited number of binary toxin- negative *C. difficile* isolates. It is difficult to explore an association between community-acquired infection and binary toxin negative *C. difficile* isolate status when other variables such as age, sex, and type of antibiotics are not also taken into account in the model. Further research is needed to evaluate whether there is an association between binary toxin isolates and CA-CDAD.

A study conducted in Hungary in 2004 also concluded that there was an association between binary toxin *C. difficile* isolates and CA-CDAD (109). Researchers analyzed 112 *C. difficile* isolates from both inpatients and outpatients to report on the different toxin types found across the country. The authors found that 79 of the isolates were positive for toxin A and toxin B, while the remaining 33 isolates were negative for toxin A and toxin B. They did not report any isolates that were negative for toxin A and positive for toxin B. Two of the isolates that were positive for both toxin A and B were also positive for the binary toxin. Further data were collected from those patients harboring the binary toxin- positive strains. It was found that both cases of CDAD were community-acquired. Neither patient had any previous history of hospitalization; however, both patients had been treated with antibiotics at the onset of diarrhea. The authors of this study concluded that binary toxin-, toxin A-, and toxin B-positive isolates may be responsible for cases of community-acquired *C. difficile* infection. The authors do not supply any information regarding how many of the isolates were community-acquired versus hospital-acquired. These further data concerning history of hospitalization and antibiotic use were collected only from the two strains that were binary toxin-positive. Because we do not know how many of the cases were community-acquired, it is difficult to determine associations between the binary toxin-positive strains and community-acquired infection. Since only two of the isolates were binary toxin-positive, most likely there was a higher prevalence of community-acquired cases that were binary toxin-negative. Also, both of the binary toxin-positive cases were receiving antibiotics at the onset of diarrhea and this is most likely the primary reason

for infection. The type of antibiotics the patients were taking may also have selected for a particular strain of *C .difficile* and this may be why these two cases had binary toxin-positive isolates. In order for the authors to form hypotheses about binary toxin positive strains and community-acquired infection, they needed to have collected further detailed information from all of the 121 cases.

Clostridium difficile-associated disease is not a problem unique to the United States; there have also been large outbreaks reported in Canada and Europe. A study was conducted in 2004 to observe the epidemiology of CDAD in a county in central Sweden (108). That study comprised three different hospitals that served the 274,000 inhabitants of the county. A total of 372 cases of CDAD arising in 335 patients from February of 1999 to January of 2000 were further investigated. CDAD was defined as diarrhea and presence of a toxin-positive *C. difficile* isolate or else presence of *C. difficile* toxin in the feces. A community-acquired case was defined as a patient with CDAD and without recent hospitalization. Ribotyping was performed on 330 of the isolates to compare strains and track nosocomial infections. They found that 59 of the 372 cases (22%) were community-acquired and 98% of the cases had a history of antibiotic use. It was not reported as to whether the 2% of cases without antibiotic use were community- or hospital-acquired. The 22% case fraction of community-acquired infections is similar to the 28% reported in a previous study in Sweden (110). All of the ribotypes were equally represented among the community- and hospital-acquired cases, except for ribotype SE17, which was found in 93% of the hospital-acquired cases. There are several factors that make it difficult to compare the prevalence of community-

acquired cases reported in this study with other studies. The first problem is that the definition of community-acquired cases is not explicit. Recent hospitalization could refer to days, months, or years. Also, it is difficult to compare studies from different countries, because of different health care systems; these may contribute to a varied demographic of patients that visit the health care facilities as well as the diagnostic tests conducted at each facility based on the patient's ability to pay. The health care facilities in Sweden are financed by the county council and medical visits are less costly for patients than in the United States; this, in turn, may lead to an increase in testing for CDAD. Studies from the United States reported a lower prevalence of community-acquired cases and this may simply be due to less frequent testing for *C. difficile* in patients without a previous history of hospitalization (82, 111).

None of the studies mentioned above were specifically designed to study CA-CDAD. Several of the studies surveyed *C. difficile* in health care facilities or outpatient clinics and from the data collected attempted to classify cases of CDAD as either community- or hospital-acquired. The prevalence of CA-CDAD varies among these studies and comparing the data is difficult due to both the differing case definitions for CA-CDAD and the differences in the study populations. It is difficult to estimate the true case fraction of community-acquired cases of *C. difficile* and this most likely will vary between jurisdictions with differing demographic profiles. However, the identification of *C. difficile* in low-risk populations, such as those with no history of hospitalization or previous antibiotic use, is important and further studies need to be conducted to better identify the sources and risk factors of these community-acquired

infections. One of the ways to identify potential sources is to identify risk factors commonly associated with these community-acquired cases.

2.2.2 Risk factors associated with community-acquired C. difficile

Results from several studies designed to examine risk factors associated with CA-CDAD have been published. The risk factors investigated include: antibiotic use, sex, age, and the use of proton pump inhibitors. Several of the risk factors, such as antibiotic use, previously known to be associated with hospital-acquired infection have also been found to be associated with community-acquired cases. Other potential risk factors, such as gastric acid suppressants, exhibit conflicting evidence as to their association with CA-CDAD.

A retrospective cohort study published in 1994 observed the epidemiology of CA-CDAD and possible risk factors associated with infection such as antibiotic use, sex, and age (82). Data were collected from the Harvard Community Health Plan, one of the largest health maintenance organizations (HMO) in New England. Patients from the five hospitals in the HMO whose primary reason for hospitalization was not psychiatric or substance abuse were included in the study. All hospitals maintained records on patients with *C. difficile* toxin assays run from April of 1988 to November of 1990. CDAD was defined as a patient with both symptoms of diarrhea or colitis and a positive *C. difficile* toxin assay. A CA-CDAD patient was further defined as CDAD with either: 1) onset of diarrhea at least 42 days after the most recent hospitalization, 2) onset within 48 hours of hospital admission, or 3) admission to a hospital exhibiting gastrointestinal symptoms and a positive *C. difficile* toxin assay result arising within 5 days. There were 51 cases

of CA-CDAD and 54 cases of hospital-acquired CDAD identified out of the 619 *C. difficile* toxin assays administered. The median age of the patients with CA-CDAD was 37 and 63% of the cases were female. There was a large percentage (43%) of patients with CA-CDAD harboring predisposing risk factors for *C. difficile* infection such as other illnesses. Among community-acquired cases, 64.7% had a history of antibiotic use and increased age was also associated with a higher risk of CA-CDAD. Sex of the patient, multiple antibiotic exposures (beyond a single episode), and HIV infection were not significantly associated with CA-CDAD. This was one of the earliest studies of CA-CDAD and illustrates that CA-CDAD shares many of the same risk factors as hospital-acquired CDAD.

A two-phase survey was conducted in Australia to determine the prevalence of *C. difficile* in stool samples submitted to a private pathology laboratory by general practitioners and to study the importance of *C. difficile* in community-acquired diarrhea (83). In Phase I, stool samples sent for microscopy and culture from patients greater than two years of age were cultured for *C. difficile*. Prior to Phase II of the survey, all practitioners who used the pathology laboratory were sent information about CDAD, its causes, and when to test for the bacterium. During Phase II, stool samples from patients greater than two years were cultured for *C. difficile* at the request of the general practitioner or if there was a history of antibiotic use in the previous four weeks. A questionnaire was sent to all general practitioners whenever any of their patients tested positive for *C. difficile*. There were 506 stool samples tested during Phase I and 580 tested during Phase II. The mean age, age range, and ratio of males to females were

similar for the two phases and none of the patients had been recently hospitalized or in a nursing home. The prevalence of *C. difficile* increased from 2.6% (13 positive stool samples) in Phase I to 10.7% (62 positive stool samples) in Phase II. The 75 positive stool samples were from 61 patients and questionnaires were sent to the general practitioners of these 61 patients. Other enteric pathogens besides *C. difficile* were isolated during the study; however, there was no statistical difference between the proportions isolated in Phase I versus Phase II. The majority of the questionnaires were returned (53 out of 61) and 85% of the patients had previously received antibiotics. The authors concluded that antibiotic use was an important prerequisite for community-acquired *C. difficile*; however, at the same time the patients must have come into contact with the bacterium. Another important issue raised in this article is that many general practitioners believed that *C. difficile* was only a problem in hospitals and nursing homes and often did not request tests for *C. difficile* on patients in the community, even those exhibiting gastrointestinal symptoms. This article was published in 1995 when there was little knowledge or data on community-acquired *C. difficile*. The increase in screening for *C. difficile* from 9% in Phase I to 42% in Phase II shows that educating general practitioners about CDAD will lead to an increase in the number of sample submitted and cases detected. There may have been misclassification of some of the cases in both phases, since other enteric pathogens were also detected alongside *C. difficile* in the stool samples, and the patients also may have been asymptomatic carriers of *C. difficile*. This would lead to inflated prevalence estimates. Also, the increase in prevalence in Phase II was most likely artificially inflated. After receiving information

on *C. difficile,* general practitioners may have been overly concerned about patients with gastrointestinal symptoms and, therefore, sent in more samples for screening even if the symptoms did not exactly match those for CDAD.

A nested case-control study was carried out in both a semi-rural and an urban population in the United Kingdom to estimate the prevalence of CA-CDAD and determine the risk factors associated with infection (84). Two cohorts of 1000 individuals, each of whom had had fecal samples submitted for clinical reasons, were randomly selected from the two distinct geographic regions, both served by a single diagnostic laboratory. Cases of CA-CDAD were defined as: 1) patients who visited their general practitioner while exhibiting symptoms of diarrhea, and 2) whose feces tested positive for *C. difficile*. Controls were defined as: 1) patients with symptoms of diarrhea who visited their general practitioner, and 2) whose feces tested negative for *C. difficile*. Three controls were matched by age and sex to each case. A questionnaire was sent to the general practitioner for each case and each control to obtain information on demographics, antibiotic use, hospitalization, and other risk factors for CDAD. Among the 2000 individuals with fecal samples submitted to the diagnostic laboratory, there was a total of 42 cases of CA-CDAD; 21 from the semi-rural region and 21 from the urban region. Cases were found to be significantly more likely than controls to have had antibiotic use and been hospitalized in the preceding 6 months; the most commonly prescribed antibiotic classes were aminopenicillins and cephalosporins. About one third (35%) of the cases had no history of either hospitalization or antibiotic use. Other risk factors that were significantly associated with CDAD were contact with infants two

years or younger in age, use of more than one antibiotic (when used), receipt of therapy for diarrheal symptoms, and an outcome of mortality. Gastric-acid suppressant drugs, number of loose stools, and specific therapies for diarrheal symptoms were not found to be significantly associated with CDAD. PCR ribotyping revealed that 60% of the isolates from the cases were PCR ribotype 1 and the isolates from the semi-rural and urban regions were different subtypes within PCR ribotype 1. The authors describe their cases as community-associated rather than community-acquired, because some of the cases had been hospitalized within the past six months and may have acquired *C. difficile* in the hospital. The finding that 35% of the cases had no previous history of hospitalization or antibiotic use and that the median age of the patients in the semi-rural population was 45 is good evidence that patients without the common risk factors for CDAD also need to be tested when they present with symptoms of diarrhea. Another interesting finding was that the median age of cases in the semi-rural region (45 years of age) was significantly lower than the median age in the urban region (73 years of age). The difference in median ages raises questions about the underlying demographics and other differences in the regions that may account for the difference in median ages. One hypothesis could be that people in the semi-rural areas have more contact with those livestock species that have been shown to be carriers of *C. difficile* (53, 54, 58, 87). One of the problems with this study was that univariate analysis was performed, because several of the questions did not generate enough response for multivariate analysis. It is, therefore, difficult to know if confounding could play a role in any of the associations. Another problem with this study was that the definition used to describe a community-

acquired case of CDAD is different than many of the other published reports. The researchers assume that all patients who visited their general practitioner with symptoms of diarrhea and had a positive fecal culture for *C. difficile* had CA-CDAD. The differences in definitions for community-acquired cases make it difficult to compare studies.

A case report published in 2008 describes a patient with community-acquired *C. difficile* with no previous history of hospitalization or antibiotic use (85). The young woman was referred to the hospital by her general practitioner after presenting with symptoms including abdominal pain, nausea, vomiting, and loose, bloody stools. Her feces tested positive for *C. difficile* toxin A and she was treated with oral metronidazole. Extensive medical history records showed that the woman had no previous history of antibiotic use or hospitalization; however, she had spent a night at the hospital three months prior to admission with one of her ill children. No previous *C. difficile* outbreaks had been recorded in the hospital and both her husband and children tested negative for *C. difficile*. The researchers felt that the previous overnight hospital stay was not responsible for her *C. difficile* infection. *Clostridium difficile* spores can survive in the environment for long periods of time and they are both heat and alcohol resistant; therefore, it is plausible that under extreme circumstances infection could occur several months after contact with bacterium. The second case presented in the article was an elderly male who acquired *C. difficile* one week after being released from the hospital. The *C. difficile* isolated from the individual was ribotype 027 and at the time there was an outbreak of 027 in the hospital. This was a case where the patient definitely was in

contact with *C. difficile* in the hospital, but did not become ill until he was at home. This second case shows how hospital-acquired infections can later surface in out-patients and these are not necessarily community-acquired. With the recent increase in *C. difficile* infections and large outbreaks it is easy to see how *C. difficile* can spread in hospitals and then appear to arise in the community. This patient could have come in contact with and spread *C. difficile* to other healthy individuals in the community either as a clinically ill individual shedding the bacteria or as an asymptomatic infected carrier. These case reports present the difference between a truly community-acquired case of CDAD and a case of hospital-acquired case of CDAD that merely surfaced in the community.

One of the hypotheses being investigated as a risk factor for CA-CDAD is the use of gastric-acid suppressant drugs (86). Gastric-acid suppressant drugs include both proton pump inhibitors (PPIs) and histamine receptor 2 antagonists (H_2RAs). Proton pump inhibitors block the hydrogen/potassium adenosine triphosphatase enzyme system, more commonly known as the gastric proton pump. This system is the final stage in gastric acid suppression and is directly responsible for secreting H+ ions (47). Histamine receptor 2 antagonists inhibit histamine action at the H_2 receptors of the parietal cells and decrease acid production stimulated by food intake (112). It has been shown in laboratory studies that the decrease in H+ ions and subsequent increase in gastric pH favors the growth or gastric-passage survival of different bacterial species such as *C. difficile* (112). In other words, it is possible that changes to the digestive tract brought about by these drugs may allow for colonization by *C. difficile* or may simply facilitate the survival of the spores (89). Gastric-acid suppressant drugs have become

one of the most widely prescribed and utilized medications in both North America and the United Kingdom (86). Not surprisingly, there have been studies published both supporting a hypothesized association between acid suppressant use and *C. difficile* in hospitalized patients (59, 90-92) and refuting it (93, 94). There exists one reported study that supports the association between CA-CDAD and the use of gastric-acid suppressants (86).

Dial et al. (2005) evaluated whether the use of gastric-acid suppressant drugs was associated with community-acquired *C. difficile* infections (86). Their data were collected from the United Kingdom General Practice Research Database (GPRD) which contains data on demographics, diagnoses, hospitalization details, and mortality arising from over 400 general practitioners. A case-control study was conducted comparing community-acquired cases (patients with a positive *C. difficile* toxin assay or clinical diagnosis, who had not been hospitalized the year prior to initial diagnosis) to ten controls matched on both the general practice they attended and their age. The two types of gastric-acidsuppressant drugs evaluated were proton pump inhibitors and histamine receptor 2 antagonists (H_2RAs). After controlling for the number of patients, age, sex, prior gastrointestinal disorders, and other co-morbidities the authors found that proton pump inhibitors and H_2RAs were associated with and increased risk of CA-CDAD. The authors also reported an increase in the overall number of cases of CDAD as well as the number of community-acquired cases from 1994 to 2004. This trend may perhaps be explained by an increased awareness of CDAD leading to more testing and therefore more diagnoses. This study provided a large sample size and controlled for many factors

that could influence the association with *C. difficile*. One of the disadvantages with this study was that the database did not provide details about the patients' symptoms. It is, therefore, not known whether the cases were symptomatic or asymptomatic. Since some of the cases were based on clinical diagnosis and not tested for *C. difficile* infection, one would assume that the patients exhibited symptoms consistent with CDAD; however, it is possible that some of the cases were misclassified.

During the study mentioned above (86), the researchers also collected data on the association of community-acquired *C. difficile* with prescribed antimicrobials (113). The same matched cases and controls were used to evaluate tetracyclines, penicillins, sulfonamides and trimethoprim, macrolides, cephalosporins and other β-lactams, and fluoroquinolones. The authors found an increased risk for CA- CDAD and antimicrobial use, especially for fluoroquinolones. However, the authors speculated that the underlying reasons for prescribing the specific antimicrobials may have been responsible for the increased risk of *C. difficile* infection rather than the antimicrobials themselves. The use of antimicrobials, especially fluoroquinolones, has been shown to increase the risk of *C. difficile* infection in hospitalized patients (35, 114), so it is not difficult to believe that this was also the case for community-acquired cases. The authors also noted that although there was a higher risk associated with CDAD and fluoroquinolone use, only 7% of the cases were prescribed fluoroquinolones and 37% were prescribed other classes of antibiotics. The low percentages of antimicrobial use among the cases provide evidence that antimicrobial use may not have been the primary underlying cause of community-acquired cases in this population.

A study conducted by the Centers for Disease Control and Prevention (CDC) in 2009 investigated the strains associated with community-acquired infection. Data arising from a multi-state surveillance program for community-acquired *C. difficile* infection (CA-CDI) involving 19 clinical laboratories in 9 states were analyzed (115). A CA-CDI case was classified as a *C. difficile*-positive stool specimen arising from an outpatient or a patient within 72 hours of admission, without a previous positive result in more than 8 weeks, or an overnight hospitalization in the last 3 months. There were 175 samples collected from patients with presumptive CA-CDI and 92 were subsequently confirmed as *C. difficile*. The positive isolates were further characterized via the *tcdC* gene, binary toxin gene (*cdtB*), toxinotyping, PFGE, and antimicrobial susceptibility. These surveillance data gave rise to a very diverse set of strains, and these strains exhibited both similarities and differences to the currently reported hospital-acquired strains. The most common strain was PFGE NAP1/ Toxinotype III, and this strain accounted for 14 of the isolates. This strain is responsible for many of the recent outbreaks in hospitals in North America and Europe. The authors noted that the prominence of this strain may be due to misclassification of cases as CA-CDI due to inaccurate records of hospital exposure. The second most prominent strain was PFGE NAP7 / Toxinotype V. This strain is most commonly found in food animals and the finding of this strain among CA-CDI, led the authors to suggest that *C. difficile* may be transmitted between animals and humans. This was a rather bold statement for the authors to make given that they only found 7 isolates that were NAP7/Toxinotype V among the 92 confirmed positive. With the large diversity among the 92 isolates we

would expect to see at least a few isolates from each of the North American pulsed field types (NAP) and toxinotypes. There were 31 PFGE types identified and 32 of the 92 isolates did not fall into one of the previously classified NAP types. The large diversity of strains may be indicative of several sources of community-acquired infection, including the environment.

2.3 Clostridium difficile *in food animals*

Recently, scientists have begun to explore the hypothesis that one of the risk factors for community-acquired *C. difficile* may be exposure to either food animals or food products. There have been a number of studies focusing on the isolation of *C. difficile* from food animals, including swine, cattle, and chickens (51-54, 58, 87, 88). *Clostridium difficile* isolates have been identified from swine showing 100% similarity to human isolates (54). *Clostridium difficile* is a suspected cause of diarrhea in calves (95) and is known to cause pseudomembranous colitis and enteritis in piglets (87, 96-99). The majority of the studies concerning food animals and food products have focused on the younger age classes where the bacterium is consistently known to occur and sometimes cause disease. There have been limited studies investigating the presence of *C. difficile* in market and slaughter age animals; however, the finding of *C. difficile* in food animals raises concern about possible food-borne and occupational exposure.

2.3.1 Clostridium difficile *in swine*

There have been many studies published concerning *C. difficile* infection in piglets. *Clostridium difficile* infection in pigs was first discovered in 1980 when gnotobiotic pigs were accidentally exposed to the bacterium (58, 116). An outbreak of

C. difficile in piglets at a farm in Canada was described in 1998 (98). Initially, only two-week-old piglets were affected, but eventually the infection spread to also affect newborn piglets. The primary post-mortem finding in the majority of the piglets was mesocolonic edema. In addition, *C. difficile* was one of several bacteria cultured from the intestines of the symptomatic piglets. *Clostridium difficile* was deemed to be responsible for the lesions in the colon of the piglets and also for the illness and mortality in the herd.

Clostridium difficile has since been found to be one of many primary agents responsible for diarrhea in piglets. *Clostridium difficile* was found in 29% of the colons of piglets in a survey in Iowa that assessed the pathogens responsible for diarrhea (97). The retrospective survey consisted of data from 100 live piglets ranging in age from 1-to-7 day- old submitted to the Iowa State University Veterinary Diagnostic Laboratory (ISU VDL) with symptoms of diarrhea. Sections of the ileum, jejunum, and colon were cultured for *E. coli*, *Salmonella*, *Enterococcus*, and *C. perfringens* and the piglets were also examined during necropsy for porcine reproductive and respiratory syndrome virus (PRRSV). Contents of the colon were tested by commercially available ELISAs for rotavirus and *C. difficile* toxins. The most common pathogen identified was rotavirus followed by *C. difficile* toxins and PRRSV. *Clostridium difficile* was identified in 29 of the 100 piglets and found in combination with other pathogens in 10 of those piglets. Mesocolonic edema was observed in all 29 of the piglets infected with *C. difficile*. When multiple piglets were submitted from the same herd with one piglet positive for *C. difficile*, 43.5% of the additional piglets were found to be positive. Information on prior

antibiotic use was available for 14 of the 29 cases and 10 of these had received antibiotics. The findings in this study are very much different than previous studies that had found *E. coli*, transmissible gastroenteritis (TGE) virus, and *C. perfringens* to be the most common causes of diarrhea in piglets. The proportional morbidity estimates in this study were not population-based and, therefore, cannot be extrapolated to all populations; however, they do provide preliminary data about the epidemiology of piglets exhibiting diarrhea and should be further investigated. It is also interesting that the majority of the piglets infected with *C. difficile* had prior exposure to antibiotics. Swine share many of the same monogastric anatomical and physiological characteristics as humans and, as a result, pigs may share some of the same risk factors for *C. difficile* infection as humans.

Clostridium difficile has been found in both symptomatic piglets with diarrhea and asymptomatic piglets (117, 118), just as it has been found in humans. A prospective case-control study was conducted to assess the association between *C. difficile* and evidence of diarrhea, mesocolonic edema, typhlitis, and colitis in piglets (117) using the same piglets as the previously mentioned study (97). The cases consisted of 100 piglets 1 to 7 days in age, submitted to the Iowa State University Veterinary Diagnostic Laboratory with symptoms of diarrhea. The controls were 29 asymptomatic piglets purchased from litters with no history of diarrhea. Contents of the jejunum, ileum, and colon were cultured for *C. difficile* during necropsy. Piglets were given diarrhea scores based on fecal staining of the perineum, hydration, and consistency of the contents in the colon. Including both cases and controls, *C. difficile* was isolated from the large

intestine in 51% of the piglets, from the small intestine in 47% of the piglets, and from the colon in 50% of the piglets. *Clostridium difficile* was isolated from the colon in 23 of the 29 control piglets and 42 of the 100 case piglets. The reason for the high prevalence of *C. difficile* in the control piglets cannot be explained in the same manner as human infants. *Clostridium difficile* is a normal component of the gut flora in infants; however, they lack the receptors for the toxin so it does not cause disease (119). As the human infant gastrointestinal tract matures, *C. difficile* is replaced by other bacteria. In piglets, *C. difficile* has been shown to cause disease and the receptors for toxin A have been identified (120). However, the presence of *C. difficile* in the gastrointestinal tract of piglets does not necessarily result in clinical symptoms. Therefore, while *C. difficile* is a necessary component cause (it is insufficient by itself), there must be other factors associated with disease. Evidence from the previously mentioned study would indicate that antibiotic use is one of these risk factors (97).

Alarez-Perez et al. (118) also examined the presence of *C. difficile* in symptomatic and asymptomatic piglets as well as the prevalence in piglets of different ages. Thirteen different farms were sampled in three regions of Spain densely populated with swine. Among the 13 farms, 10 had a previous history of neonatal swine diarrhea and served as the case farms, whereas the 3 remaining were clinically free of diarrhea and served as the control farms. Rectal swabs were taken from 541 piglets ranging in age from 1-to-7 days-old and 239 piglets ranging in age from 1-to-2 months-old. Among the 1-to-7 day-old piglets, 287 were asymptomatic and 254 were symptomatic (diarrhea). Among the 1-to-2 month-old piglets, 187 were asymptomatic and 52 were symptomatic.

C. difficile was isolated from 140 of the 541 (25.9%) rectal swabs from the 1-to-7 day-old piglets and at least one asymptomatic piglet tested positive for *C. difficile* from each farm, including the clinically free farms. *Clostridium difficile* was not isolated from any of the 1-to-2 month-old piglets. Among the 1-to-7 day-old piglets, *C. difficile* was isolated from 82 (28.6%) of the asymptomatic piglets and 58 (22.8%) of the symptomatic piglets. As in the previous study by Yaeger et al. (117), this study also provided evidence that *C. difficile* can be isolated from both symptomatic and asymptomatic piglets, and that *C. difficile* infection in piglets was similar in nature to infection in human infants. *C. difficile* was not isolated from any of the 1-to-2 month-old piglets in this study, and as such it appears that *C. difficile* was replaced by other bacteria in the gut as the piglets matured (similar to humans).

The majority of the literature on *C. difficile* in swine has focused on the disease in piglets. In 2005, an article was published describing an outbreak of *C. difficile* in postparturient sows in Croatia (121). That outbreak took place in a large outdoor commercial swine operation consisting of 2,949 sows and occurred between January and March of 2003. All of the sows had previously been vaccinated against parvovirus, erysipelas, leptospirosis, *C. perfringens* C, *E. coli* and pseudorabies. Sows were routinely tested and treated for mastitis metritis agalactia (MMA) disease complex. Among 614 sows suffering from and treated for MMA, 122 (19.9%) showed signs of respiratory distress and 81 (13%) died. In comparison, only 0.4% of the sows not suffering from or treated for MMA died. Four sows, representative of all the mortalities, were sent for necropsy, bacteriological, and serological investigation. A finding of

mesocolonic edema, lesions, *C. difficile* isolates, and *C. difficile* toxins led the researchers to conclude that all 4 mortalities were a result of *C. difficile* infection. Other possibilities such as *E. coli*, *Salmonella*, PRRS, and classical swine fever were excluded as a result of the necropsy, bacteriological, and serological tests. Postparturient death was 6.2% during the outbreak from January to March in comparison to 2.4% before January and 2.6% after March. The sows with MMA had given birth to a similar number of litters to those without MMA; however, the sows treated for MMA were generally younger than the non-diseased herdmates. This study demonstrates that older swine are at risk for *C. difficile* infection; however, other risk factors may be involved with the infection. The risk factors involved with this outbreak include antibiotic use (treatment of MMA with enrofloxacin), environmental conditions, and a weakened immune system. These sows were housed outdoors during the winter, which most likely put stress on the animals. The sows also had weakened immune systems from recently having given birth. It is also interesting to note that there was no increase in loss of piglets during the outbreak of *C. difficile* in the herds; this demonstrates further that the mere presence of the bacteria alone was not enough to cause disease.

2.3.2 Transmission between species

The finding of *C. difficile* in food animals has led researchers to explore the possibility of transmission between animals and humans. Most studies providing evidence of transmission have focused on the similarities and differences among gene or toxin subtypes found in animals and humans (53, 54, 100-102). There have been several studies exploring the subtypes of *C. difficile* in swine populations and some of these

studies have also compared swine isolates to human isolates (122, 123). The most common ribotype and toxinotype found among swine isolates is ribotype 078, toxinotype V (101, 122). This strain is characterized by a 39 base-pair deletion in the *tcdC* gene, which is the negative regulatory gene for toxin A and toxin B production, presence of the binary toxin gene, and presence of both the toxin A and toxin B genes. No studies have been published investigating the possibility of *C. difficile* transmission through exposure to food animals; however, one study has provided data supporting occupational exposure of health care workers to *C. difficile* in clinical settings (124).

One of the first papers published comparing types of *C. difficile* found in humans and animals, to explore the possibility of transmission between species, compared ribotypes from clinical human cases to canine, equine, feline, and bovine isolates collected from previous studies (100). A total of 133 isolates comprised of 92 canines, 21 equine, 20 human, 1 feline, and 1 bovine isolate were analyzed using ribotyping and PCR to identify the toxin genes. While there were 23 distinct ribotypes identified, there typically were 1 or 2 dominant ribotypes for each animal species. The single bovine isolate was the same ribotype as a ribotype found among the equine isolates and the one feline isolate was a ribotype found among the canine isolates. There were 7 different ribotypes identified among the human isolates and 2 of these ribotypes were also found among the canine and equine isolates. There was a large diversity among the ribotypes for the equine isolates, whereas 77% of the canine isolates belonged to the same ribotype. This dominant canine ribotype was found in 20% of the human isolates. The toxin A and B genes were identified in all of the human and equine isolates and in 69%

of the canine isolates. The remaining canine isolates were negative for both the toxin A and toxin B genes. This study was the first to report that there were similar ribotypes found among humans and animals and that interspecies transmission was therefore a possibility. The finding of similar ribotypes among dogs and horses is of concern, because these are companion animals and a large portion of the human population comes in contact with these animals on a daily basis. In addition, dogs are often used for non-medicinal therapy purposes in hospitals and nursing homes where patients tend to have additional known risk factors for *C. difficile* infection. Several studies have been published exploring the presence of *C. difficile* in companion animals and in therapy dogs (125-128).

A study published in 2007 surveyed the relative proportion of ribotypes among swine, bovine, canine, equine, and human isolates from various geographic areas (101). A convenience sample (samples were selected based on ease of sampling) of swine isolates were collected from piglets in Iowa, Ohio, Montana, North Carolina, and Utah and bovine isolates were collected from calves less than 2 months of age in Arizona. Equine isolates were collected from Kansas, Arizona, and Canada and canine samples were also collected from Ontario, Canada. Human isolates were from two hospitals in Colorado. Information on signalment was not provided for the equine, canine, or human samples. A total of 232 *C. difficile* isolates including 33 bovine, 12 canine, 20 equine, 144 swine, and 23 human isolates were ribotyped and tested for the presence of toxin A and toxin B. Nineteen different ribotypes were identified, with the largest diversity of ribotypes among the human, equine, and canine samples. Ribotype 078 accounted for

94% (31/33) of the bovine isolates, 83% (119/144) of the swine isolates, 5% (1/20) of the equine isolates, 4.4% (1/23) of the human isolates, and none of the canine isolates. The majority of the canine isolates were type 010, the equine isolates were type 015, and the human isolates were type 020. There were 4 different ribotypes found among the swine isolates and 12 different ribotypes found among the human isolates. The lack of diversity in ribotypes among the swine isolates is interesting, because swine were the largest group sampled and had the most diverse geographic representation. This study found that there was a larger diversity of ribotypes among human isolates than the swine isolates and there was no association between the strains found in swine and in humans.

A second study comparing human and animal isolates found that the number of toxinotype V isolates had increased in recent decades and when analyzed by PFGE there was 100% similarity between the human and swine isolates (54). Past isolates were analyzed from 1984 to 2001 in the Hine Veterans Affair (VA) Hospital database and compared to recent isolates from hospital outbreaks sent to the Center for Disease Control (CDC) from 2001 to 2007. Clinical information was obtained for all the recent cases. Cases were considered community-acquired if symptoms began less than 48 hours after admission and it had been more than 12 weeks since they were discharged. Hospital-acquired cases were patients with symptoms beginning more than 48 hours after admission or less than 4 weeks after discharge. Patients receiving antibiotics within 30 days of symptom onset were considered to be exposed to antibiotics. Swine isolates were obtained from piglets from North Carolina, Iowa, Texas, Utah, Ohio, and Arizona from 1999 to 2005 and bovine isolates were obtained from 1-day to 6-week-old

calves in Arizona from 2003 to 2005. Positive isolates were further analyzed by PCR for the *tcdC* gene deletion, binary toxin gene, toxin A gene, and toxin B gene. Toxinotyping and PFGE were also conducted to compare the isolates. Seven toxinotype V isolates were identified among the more than 6,000 isolates of *C. difficile* from the Hine VA Hospital and 8 toxinotype V isolates were identified from the 620 recent cases sent to the CDC from hospital outbreaks. They found a significant ($P<0.001$) difference between the 7 past and 8 recent toxinotype V isolates. Among the recent cases, 38% were community-acquired and 88% were exposed to antibiotics. Among the past cases with available data, 3 out of 5 cases (60%) were community-acquired and all 4 of the cases were exposed to antibiotics. PFGE analysis revealed three different clusters, each containing at least one human and swine isolate with 100% similarity. One of the major limitations of this study is that the past and recent populations are not comparable due to varied population sampling strategies. The past isolates arose from a database of a VA hospital, whereas recent isolates were sent to the CDC from outbreaks. It would be interesting to look at the number of toxinotype V isolates from the Hine VA Hospital from 2001 to 2007 and compare this number to the seven cases from 1984 to 2001. The researchers also tried to link the increase in toxinotype V in humans to the emergence of toxinotype V in animals. There is no evidence that toxinotype V has only recently been found in animals, and the emergence of this strain in animals is most likely an artifact of increased testing in animals. A major limitation in this study is the small number of toxinotype V isolates. With only 15 total toxinotype V isolates there is little statistical power to compare toxinotype V with the risk of community-acquired infection. The

human and swine isolates used in this study were also not from the same geographical regions or catchment areas and, therefore, it is difficult to suggest that transmission between host species is a reason for the similar toxinotypes.

The discovery of *C. difficile* in food animals is not restricted to the United States. International studies have found both similar prevalence and types of *C. difficile* and some authors have suggested that any differences between nations may be related to demography and geography. One study conducted in Slovenia reported survey data from swine and cattle farms tested for the presence of *C. difficile* and compared these isolates to those found on U.S. farms (53). Samples were collected from 257 piglets from three different farms and 56 calves from two different farms. All piglets and calves that were sampled exhibited symptoms of diarrhea. *Clostridium difficile* was isolated from 133 (51.8%) of the piglets and 1 (1.8%) of the calves. The single calf isolate was negative for both the toxin A and toxin B genes, but positive for the binary toxin and belonged to toxinotype XIa, ribotype 033. The 102 piglet isolates from two of the farms were found to be toxinotype V, whereas the 31 piglet isolates from the third farm were toxinotype 0. Isolates belonging to toxinotype 0 are positive for both toxin A and toxin B, but negative for the binary toxin. Toxinotype 0 strains have been found in 150 different ribotypes, but all of the isolates from this study belonged to the same ribotype that had not previously been identified. Toxinotype V isolates have been classified as either ribotype 078 or 066. All of the toxinotype V isolates in this study belong to ribotype 066. The finding of ribotype 066 in piglets in Slovenia contrasts with data from the United States that have found the majority of isolates from piglets are ribotype 078.

Toxinotype V was found in piglets in the U.S. and Slovenia and it is possible that some of the studies from the U.S. that only compared toxinotypes and not ribotypes contained ribotypes other than 078. The differences in ribotypes may also be explained by variations in the geographic distributions of different strains.

Piglets were sampled from several farms in the Netherlands to describe the different *C. difficile* types in the population (122). Forty-eight piglets between one to four days of age and suffering from diarrhea were sampled from two farms for the presence of *C. difficile*. Another 272 asymptomatic piglets were sampled as controls from seven large farms that were members of the European Pig Producers Association. After nine months, the two case farms were still experiencing cases of diarrhea and 31 more samples were taken from symptomatic piglets. The number of positive isolates from each of the asymptomatic and symptomatic group of piglets was not provided. All of the positive *C. difficile* isolates were found to be ribotype 078, toxinotype V. These isolates were similar to those isolates characterized and arising from hospitalized patients in the Netherlands (129). In order to test the relatedness of the isolates, 11 of the swine isolates and 21 of the human isolates were analyzed using multi-locus variable-number tandem-repeat analysis (MLVA) (122,129). All of the isolates were found to be genetically related and in one of four clonal complexes. One of the clonal complexes contained both human and swine isolates and two pairs of human and swine isolates were found to be 100% homologous. It is interesting that both swine and human isolates in the Netherlands were found to be ribotype 078, toxinotype V; however, the swine and humans are not from the same population so it is difficult to make any

meaningful comparisons between the isolates. The authors have merely pointed out that the swine and humans colonized with these isolates may have been exposed to a common point source. Another concern with this article is that the number of positive isolates from each of the symptomatic and asymptomatic piglets is not given. We do not know if the 11 isolates that were analyzed by MLVA were the only isolates recovered or if it was a sample. We also do not know how many of the isolates came from each of the two case farms and the seven control farms. A table showing the antimicrobial susceptibility testing from the case farms only provides data from 1 isolate from farm one and 4 isolates from farm 2. In addition, no information is provided on the number of isolates typed and whether all the isolates were ribotype 078, toxinotype V or if this was simply the majority.

The human samples mentioned above were collected during a study in the Netherlands from January 2005 through January 2008 (130). A total of 1,687 isolates from patients with CDAD were typed at the National Reference Laboratory at Leiden University Medical Center. Questionnaires were sent to patients with CDAD to study the risk factors associated with each of the different ribotypes. The most common ribotypes identified were type 027 (17%, 289 isolates), type 014 (10%, 173 isolates), type 078 (9%, 150 isolates), and type 001 (2%, 29 isolates). During the study period, the proportion of ribotype 078 isolates increased from 3% to 13% and the proportion of ribotype 027 isolates decreased from 27% to 1%. Approximately 40% of the questionnaires were returned from each of the groups of patients; type 027, type 078, and all other ribotypes. Odds ratios were reported for comparisons between ribotype

078 to all other ribotypes (except 027), ribotype 078 to ribotype 027, and ribotype 027 to all other ribotypes (except 078). When comparing patients with ribotype 078 to the other ribotypes (except 027), patients with 078 were more likely to have used fluoroquinolones (OR 2.17 (1.06, 4.44)), but were less likely to have other diseases (OR 0.44 (0.20, 0.95)). Comparing patients with type 078 and type 027, patients with 078 were generally younger and cases were more often community-acquired (OR 2.98 (1.11, 8.02)). The risks of severe diarrhea and mortality were similar for type 078 and 027; however, patients with 078 generally had a less complicated course of treatment (OR 0.20 (0.04, 0.91)). Patients with type 027 generally were older (OR 1.94 (1.35, 2.79)) and cases were more frequently health care- associated when compared with ribotypes other than 078 (OR 3.85 (1.82, 8.15)). Patients with 027 isolates also were more frequent users of cephalosporins (OR 1.66 (1.03, 2.67)) or fluoroquinolones (OR 1.69 (1.00, 2.88)), exhibited symptoms of severe diarrhea (OR 1.65 (1.02, 2.67)), had a more complicated course of treatment (OR 1.53 (0.81, 2.88)), and resulted in mortality (OR 1.57 (0.74, 3.35)) when compared to ribotypes other than 078. A random selection of 51 of the type 078 isolates revealed that all of the isolates were positive for: 1) toxin A, b) toxin B, c) the binary toxin, d) had a 39 base pair deletion in the *tcdC* gene, and e) were toxinotype V. As mentioned, these human isolates had a high degree of relatedness to the swine isolates collected from two farms in the Netherlands. The majority of the samples surveyed were from hospitals and regional laboratories that submit samples on a monthly basis. The remaining samples were from facilities that only submit samples for patients with severe CDAD or when there is an outbreak of CDAD. When the results

from these two groups were analyzed separately, similar results were found so this likely did not introduce bias into the study. Although questionnaires were not returned for all the patients, an even proportion was returned for each ribotype group. This study provides evidence that ribotype 078, toxinotype V is found among human cases of CDAD and the proportion of patients with this type appears to be increasing. One of the previously mentioned studies conducted in the United States also found that the number of toxinotype V cases is increasing (54). The relationship between human cases of ribotype 078, toxinotype V and swine cases is not yet known, but they have a high degree of relatedness, which raises the specter of either transmission between the two species or else infection from a common source.

Ribotyping, toxinotyping, and PFGE are the most common techniques used to analyze and compare isolates; however, other techniques have also been used. One of the other published techniques involved triple-locus sequence analysis of the toxin regulatory genes *tcdC*, *tcdR*, and *cdtR* and also analysis of deletions in the *tcdC* gene to compare human, swine, and equine isolates (102). The *tcdC* gene is a negative regulatory gene for the production of toxin A and B, the *tcdR* gene is a positive regulatory gene for the production of toxin A and B, and the *cdtR* gene is a positive regulatory gene for the binary toxin. The *tcdC* gene, *cdtR* gene, and a 473-base pair fragment of the *tcdR* gene were amplified for 53 isolates chosen based on the size of their *tcdC* gene deletion (102). These isolates included 38 human, 4 swine, and 11 equine samples. There also were 4 control strains from human clinical cases included, each with known sequenced *tcdC* genes. Deletions in the *tcdC* gene were identified in

43 (75.4%) of the 57 isolates and there were 4 different deletions identified including an 18-, 36-, 39-, and 54-base pair deletion. Ten different clusters were found using triple-locus sequence analysis varying from 1 to 20 nucleotide differences in the isolates. The majority of the sequences with the same *tcdC* gene base pair deletion clustered together; however, some of the isolates with an 18-base pair deletion also clustered with isolates with no deletion in the *tcdC* gene. All of the isolates with a *tcdC* gene deletion also contained the binary toxin. Three clusters of isolates had specific alleles in the *tcdC*, *tcdR*, and *cdtR* genes that were not found in any of the other isolates. The three clusters were the toxin A- negative, toxin B-positive isolates belonging to toxinotype VIII, the isolates with a 39-base pair deletion in the *tcdC* gene belonging to toxinotype V, and the isolates with the 54-base pair deletion in the *tcdC* gene. These three clusters each had a separate lineage and appeared to have evolved separately, whereas the strains with a 36-base pair deletion, 18-base pair deletion, and the wild type with no deletion had overlapping lineages. This is interesting, because it appears that the toxinotype V isolates did not evolve from one of the other strains of *C. difficile*, although it has only recently been recognized and may be increasing in prevalence. Toxinotype V isolates are found in both humans and animals, but do not share a similar lineage with any of the other toxinotypes in this study identified from humans, swine, or equine. The findings from this study provide evidence to support the hypothesis that toxinotype V isolates originated from a common source, such as the environment, rather than originating in either human or animal hosts.

The prevalence of *C. difficile* in food animals was estimated in a study in Austria in 2008 (123). Fecal samples were collected from 67 cattle, 61 swine, and 59 broiler chickens from 65 abattoirs from March to July. Positive isolates were further characterized by ribotyping, as well as by PCR for the presence of the binary toxin, toxin A, and toxin B genes. *Clostridium difficile* was isolated from 8 of the 187 fecal samples including 3 (4.5%) from cattle, 2 (3.3%) from swine, and 3 (5%) from broiler chickens. All eight of the positive samples were of a different ribotype and included: 126 and A-50 in the swine; Al-225, 420, and 014/0 in the cattle; and Al-79, 001, and 446 in the chickens. Based on previous work, the most common ribotype found among cattle and swine is type 078 (101). Ribotype 126, as found in one of the swine samples in this study, is very closely related to ribotype 078. It is important to note in this study that these samples were taken from asymptomatic animals at slaughter. This is the most likely explanation for the lower prevalence of *C. difficile* than in previous studies (53, 87). Previous studies have focused on piglets and calves with diarrhea, whereas this study sampled older animals that were headed to slaughter. The lower prevalence of *C. difficile* in animals headed to slaughter could be seen as evidence of a lowered risk of transmission of *C. difficile* from animals to humans through food.

Although no studies have investigated the possible transmission of *C. difficile* from food animals through occupational exposure, a study published in 2009 evaluated the occupational exposure of health care workers to patients with *C. difficile* infection (124). Health care workers from 4 different internal medicine wards at an academic hospital were evaluated for skin and fecal carriage of *C. difficile*. One of the 4 wards

had patients with confirmed *C. difficile* infection, whereas the remaining three wards did not contain any patients with diarrhea or confirmed cases of *C. difficile*. Information on profession, antibiotic use, contact with symptomatic *C. difficile* infected patients, and bowel movements was collected from participating health care workers; as well as hand swabs, swabs from the lower abdominal region, and fecal cultures. *Clostridium difficile* was not isolated from any of the hand or abdominal swabs. A total of 30 fecal samples were collected (13 from the *C. difficile* ward and 17 from the control wards) and 3 (23%) from the *C. difficile* ward and 1 (6%) from the control wards tested positive for *C. difficile*. Unfortunately, the researchers did not type the isolates found among the health care workers, so it is not known whether the health care workers and patients were infected with the same strains of *C. difficile.* Although the data on the strains is lacking from this study, it suggests that occupational exposure to *C. difficile* may occur in the clinical setting.

2.4 Clostridium difficile *in retail meat samples*

In contrast to the low prevalence of *C. difficile* found in slaughter age animals, *C. difficile* has been more readily, if inconsistently, identified in surveys of retail meats (48, 50, 103). The reported prevalence of *C. difficile* in retail meats has ranged from 12% to 40%. Many of these studies have covered small geographic areas and this may partially explain the wide distribution of prevalences. The published studies have included both raw and ready-to-eat products from various sources including swine, cattle, and chickens. The finding of *C. difficile* in retail meat raises the possibility of the bacteria to be classified as a foodborne pathogen. *Clostridium difficile* spores can survive under

extreme heat and will readily survive when meat is cooked to the recommended temperatures (56)

One of the first published studies evaluating the presence of C. *difficile* in retail meat samples was conducted in Canada (56). Fifty-three ground beef and 7 ground veal samples were collected from 4 grocery stores in Ontario and 1 in Quebec. The 57 samples from the 4 stores in Ontario and the 3 samples from the store in Quebec were collected from January to October in 2005. *Clostridium difficile* was isolated from 12 (20.0%) of the 60 retail meat samples including 11 (20.8%) ground beef samples and 1 (14.3%) ground veal sample. Eight of the 12 isolates were of a ribotype that had not been previously identified in the authors' laboratory and were designated ribotype M13. Isolates belonging to this ribotype were positive for the binary toxin, toxin A, and toxin B genes, had an 18-base pair deletion in the *tcdC* gene, and were toxinotype III. The molecular characteristics of ribotype M13 are the same as those for the virulent human strain ribotype 027, but the ribotype patterns were distinctly different. PFGE analysis of these ribotype M13 strains resulted in a pattern 80% similar to strain NAP1, which is also the PFGE classification of the virulent strain. The three remaining isolates belonged to ribotypes 077, 014, and M26. Ribotypes 077 and 014 have been identified as human pathogenic strains. Previous to this study, *C. difficile* had been incidentally identified in a commercial raw meat diet intended for dogs (131) and in a study on "blown package" spoilage of vacuum-packed meats (132), but had not been identified in retail meat intended for human consumption. One of the major disadvantages of this study is that the samples were selected by convenience. The samples originated from 5

different grocery stores; however, the majority of the samples originated from just 4 of the stores. The 8 samples that were identified as ribotype M13 all originated from the same store, which suggests cross-contamination at the packaging or distribution level. The only other ribotype that was found in more than one sample was type 077 and this ribotype was found in two ground beef samples from the same store. The prevalence of *C. difficile* in retail meat samples appears to be high based on previous incidental findings of *C. difficile* in raw meat (131, 132). This higher prevalence may be explained by the isolation technique used in this study. The isolation technique included a 2-week enrichment process prior to plating and this long enrichment process could have helped detect low levels of spores. However, since this was the first study to evaluate the prevalence of *C. difficile* in retail meat intended for human consumption, it is difficult to know if this was an accurate representation or if the prevalence was high due to a potentially post-harvest (or during processing) contaminated batch in one of the stores.

A study evaluating the presence of *C. difficile* both in raw and ready to eat retail meat samples conducted in 2007 reported a much higher prevalence (48). Samples were collected from three retail stores in Tucson, Arizona on three different dates in one month intervals from January to April. Samples included 26 ground beef, 7 ground pork, 9 ground turkey, 13 pork sausage, 10 pork chorizo, 7 beef summer sausage, and 16 pork braunschweiger sausage. Products with different sell-by dates were selected for each type of meat sample to observe associations with production date. Both a heat-shocked and non-heat-shocked culture technique was used to isolate *C. difficile*. Interpreting both culture techniques in parallel, *Clostridium difficile* was isolated from

37 (42.0%) of the 88 retail meat samples, including 13 (50%) ground beef, 1(14.3%) summer sausage, 3 (42.9%) ground pork, 10 (62.5%) braunschweiger sausage, 3 (30%) chorizo, 3 (23.2%) pork sausage, and 4 (44.4%) ground turkey. Positive samples originated from both the heat-shocked and non-heat-shocked isolation techniques. All samples that were classified as negative were negative for both culture techniques; whereas samples classified as positive were positive for at least one of the culture techniques. None of the samples were positive for both culture techniques. No associations were found with the meat processor, sell-by date, store, or month sampled. Twenty seven (73.0%) of the isolates were ribotype 078 and toxinotype V and the remaining 10 (27.0%) isolates were ribotype 027 and toxinotype III. The C. *difficile* strains isolated from retail meat in this study are interesting, because ribotype 078 is the most common strain isolated from food animals including pigs and cattle, whereas ribotype 027 is the virulent strain associated with increased human outbreaks both in North America and Europe. One of the key pieces of information missing in this article are the data on the number of samples collected from each retail store and retail store data for the positive isolates. The authors state that there were no associations between positive isolates and the processor, sell-by-date, store, or month sampled; however, none of these data are provided. The prevalence estimated in this study was much higher than that reported in Canada. Reasons for this might include: different isolation techniques, different types of meats sampled, different processing methods, and geographical variation. The isolation technique used in this study included a 3-day enrichment process prior to plating; whereas the study in Canada used a 2-week enrichment. It

would seem that the two week enrichment used in the Canadian study would be a more sensitive method than the 3-day enrichment used in this study; however, the other difference in the isolation techniques involved the heat- or alcohol-shock treatments. This study used a heat-shock procedure, whereas the Canadian study used an alcohol-shock procedure. Another study evaluated different isolation techniques and reported that alcohol-shock was more effective than heat shock at isolating *C. difficile* (133). Based on the shorter enrichment time and use of heat-shock instead of alcohol-shock, it does not appear that the isolation techniques used in this study are responsible for the higher apparent prevalence. This study sampled a variety of different meats, whereas the Canadian study only sampled ground beef and ground veal. However, when we observe only the ground beef samples this study estimated prevalence at 50%, whereas the Canadian study yielded 20% prevalence. Even if this study only evaluated the ground beef samples, it would still have estimated a much higher prevalence of *C. difficile*.

There have been several studies conducted outside of North America evaluating the presence of *C. difficile* in retail meat samples. An extension of report described earlier, one of these studies was conducted in Austria in 2008 (123). Meat samples, including 51 beef, 27 pork, and 6 chickens, were collected from 11 retail outlets from February to April of 2008. The beef and pork samples included trim samples as well as ground samples. *Clostridium difficile* was not detected in any of the meat samples. One of the problems with this report is that the geographic distribution of the source of the retail meat samples was not reported. The samples were collected from 11 retail outlets, but it is not known if these samples were representative of all of Austria or only a

specific region. The data on the distribution of samples from each retail outlet are also not provided. The finding of *C. difficile* in retail meat may be a result of contamination at packaging or distribution and if all of the samples came from the same packaging or distribution plants then the samples are not representative of the broader population. It has been suggested that the prevalence of *C. difficile* in retail meat is higher in North American than in Europe. The prevalence and strains of *C. difficile* in food animals are similar in North America and Europe and if animals were the source of the contamination in retail meat a similar prevalence would be expected in North America and Europe. The isolation techniques used in this report were comparable to those used in North America and do not appear to be responsible for poor sensitivity of *C. difficile* detection. The samples were analyzed using a direct plating technique in conjunction with a technique that included a 2-week enrichment step prior to plating. Studies published from the United States have included both a 1-week and 2-week enrichment step prior to plating (48, 56). Seasonality may be another reason for the lack of detection of *C. difficile* in the samples in this study. Samples in this study were only collected from February to April. Other studies have suggested a possible season trend in detection from retail meat samples (50).

A low prevalence of *C. difficile* in retail meat was also found during a pilot study conducted in Sweden in 2008 (134). Samples were collected from randomly selected retail shops in Uppsala, Sweden from April to September. A total of 82 ground meat samples were collected and comprised 32 beef, 11 pork, 12 mixed beef and pork, 7 sheep, 2 moose, 1 calf, 2 poultry, 5 beef hamburger meat, 1 mixed beef and pork

hamburger meat, 1 cooked hamburger meat, 2 cooked poultry sausage, and 6 cooked beef or pork sausage. Several different isolation techniques were used including direct plating on selective agar, a 10-to 12-day enrichment process followed by plating to selective agar, and an alcohol-shock treatment followed by plating to selective agar. *Clostridium difficile* was isolated from only 2 (2.4%) of the 82 retail meat samples. The 2 positive samples originated from ground beef; however, they were purchased from separate stores in different months (one of the samples was purchased in May and the other in September). Both of the samples were isolated using the 10- to 12-day enrichment process followed by plating onto selective agar. The positive isolates were positive both for toxin A and toxin B. The prevalence of *C. difficile* in retail meat was much lower in this report compared to studies reported from the United States and Canada (48, 56). The low prevalence estimated in this report is more comparable to the Austrian investigation that failed to detect any *C. difficile* in retail meat (123). The Swedish researchers used a culturing technique that included a 10- to 12-day enrichment process; this is comparable to the technique used by Rodriguez-Palacios et al. (56), therefore the isolation technique cannot be solely responsible for the lower prevalence.

Initial studies on the prevalence of *C. difficile* in retail meat vary in estimates of prevalence from 0 to 40%. It is not known whether differences in estimates are due to culturing techniques, the types of meat sampled, limited sampling distribution, or geographic distributions. A study conducted in Canada in 2009 set out to move beyond these limitations by analyzing the prevalence of *C. difficile* in retail meat using a broad government sampling infrastructure, 3 different culture techniques, and evaluating

month to month variability (50). Samples were collected as part of an active surveillance program for the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS). Ground beef and veal chops were purchased from two randomly selected census divisions per week from various retailers throughout Canada. A total of 214 retail meat samples, including 149 ground beef and 65 veal chops, collected from 210 different retailers from January to August 2006 were tested using the three different culture techniques. All of the culture techniques included a 7-day enrichment, an alcohol-shock treatment, and plating onto solid agar. The three techniques were a rinsate sample enriched in *Clostridium difficile* moxalactam norfloxacin broth supplemented with 0.1% sodium taurocholate (TCDMNB) and plated on *Clostridium difficile* moxalactam norfloxacin agar (CDMNA); a meat sample enriched in TCDMNB and plated on CDMNA; and a meat sample enriched in cycloserine cefoxitin fructose broth supplemented with 0.1% sodium taurocholate (TCCFB) and plated on blood agar. *Clostridium difficile* was cultured from 13 (6.1%) of the 214 samples including 10 (6.7%) ground beef and 3 (4.6%) veal chops. Analyzing the data by month, *C. difficile* was more commonly isolated in January and February (11.5%) than in March through August (4%). When comparing the three different culture techniques, isolation rates varied from 1.4 to 2.3% and there was little or no agreement or reproducibility between the techniques. The reported sensitivities of the culture techniques were low and varied from 23 to 39%. The broad government sampling infrastructure used in this study represents a great improvement over the convenience samples used in the previous studies. However, this study did not address

all of the limitations of past studies and also introduced some new limitations of its own. One of the limitations with this study was the way the prevalence data were presented. The authors presented an overall prevalence of 6.1%; however, this was interpreting the results of the three techniques in parallel, which would falsely inflate the sensitivity. There were no samples that were positive for more than one culture technique. When presenting estimates of prevalence, the sensitivity and specificity of the culture techniques need to be taken into account. In this study, it would be more accurate to present the prevalence of each technique separately. This would reduce the prevalence estimates to 1.4, 1.9, and 2.3% for each of the three techniques, respectively. Also, the sensitivity of the three techniques was calculated based on parallel interpretation of the culture techniques and an overall prevalence of 6.1%. Sensitivity and specificity estimates of a culture technique need to be calculated using comparison to a gold standard technique or through prior estimates and Bayesian estimation. In this article the authors used their own data to estimate sensitivity and no data was known about the number of true positives, false positives, true negatives, and false negative among the retail meat samples. Another problem with the three different culture techniques is that the authors did not address the differences found when compared with previous studies. Some of the differences in other studies that needed to be addressed were differences in enrichment periods and also the difference between alcohol-shock treatment and heat-shock treatment. The culture techniques in this paper compared different enrichment broths and selective agars as well as looked at the difference between using the meat samples and a rinsate sample. The advantage of using a rinsate sample is that the meat

samples are added to peptone water and then mixed in a stomacher to create a homogenous sample. It would be interesting to determine whether rinsate samples tested in duplicate would yield replicated results. Another limitation of this study was that the researchers set out to analyze the month-to-month variability of *C. difficile* in retail meat samples, but then only collected samples from January to August, with a disproportionate number of samples collected during a few months. The number of samples collected each month ranged from a high of 73 samples collected in May to 0 samples collected in July. The authors believed that there was a possible seasonal difference in *C. difficile* prevalence in retail meat, but it is difficult to support this hypothesis when samples were not collected for the entire year and were disproportionately collected each month during the sample period. In order to test if there is a significant difference in *C. difficile* in retail meat during the winter seasons it would also be helpful to have samples from more than one year. It is possible to have a significant difference in season for one year; however, this may easily be caused by other confounding factors and may not hold up over several years.

Several studies have focused on *C. difficile* in retail chicken. One study conducted in Canada evaluated the prevalence and types of *C. difficile* in chicken legs, thighs, and wings purchased from retail stores (135). A total of 203 samples (111 thighs, 72 wings, and 20 legs) were collected as part of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) from November of 2008 to June of 2009. Samples were cultured using a direct plating method on selective agar as well as a method that involved a 48-hour enrichment process and an alcohol-shock treatment,

followed by direct plating to selective media. *Clostridium difficile* was isolated from 26 (12.8%) of the 203 samples including 10 thighs (9.0%), 13 wings (18%), and 3 (15%) legs. The 26 positive isolates were all ribotype 078, positive for toxin A, positive for toxin B, and positive for the binary toxin. The prevalence of *C. difficile* found in the chicken samples in this study was similar to the 20% found for retail beef and veal in a previous Canadian study (56). The prevalence was higher than that found in retail meat in Austria and Sweden (123, 134) but lower than the prevalence found in a study in Arizona (48). The studies conducted in Sweden and Austria included a handful of chicken samples and did not detect *C. difficile* in any of these (123, 134). This Canadian report was the first to look at a larger sample size of retail chicken samples and the results were similar to other studies that found *C. difficile* in retail meat and also found strains commonly found in food animals (135). However, ribotype 078 is not one of the strains commonly isolated from chicken fecal samples (51). A study in Slovenia found that the majority of the isolates from chicken fecal samples were toxinotype 0 and none of the isolates were toxinotype V, the toxinotype associated with ribotype 078 (51). Another study in Austria found ribotypes AI-79, 001, and 446 among the chicken fecal samples (123). The Austrian study used both an enrichment procedure and direct plating procedure for the isolation of *C. difficile*. All of the positive samples were isolated using the enrichment procedure with a detection limit of less than 10 spores per gram of sample, which would suggest that the chicken samples were contaminated with a low level of *C. difficile*. The previously mentioned study conducted in Canada that found a similar prevalence of *C. difficile* in ground beef and veal used the same isolation

procedure (50). The isolation procedure in these studies is different than those used in the other studies in Austria, Sweden, and the United States and may be a major reason for the differing prevalence.

The finding of *C. difficile* in calves has raised concern about *C. difficile* in retail beef products as well as in milk. Another study conducted in Austria evaluated the presence of *C. difficile* in ground beef (and pork samples) as well as in raw milk samples (136). One hundred ground meat samples were purchased from retail stores from July 2007 to February 2008. Raw milk samples were taken from bactofugates in two large dairies from February to June of 2008. Bactofugation is a centrifugation process used at dairies to reduce the number of spores in the raw milk before heat treatment. Prior to culturing the meat and milk samples the researchers tested four different isolation procedures using spiked retail meat and raw milk samples. The only procedure that supported the growth of *C. difficile* was enrichment in CDMN broth followed by plating on the corresponding selective agar (CDMN). The other isolation methods resulted in the isolation of other *Clostridium* species including *bifermentans* and *glycolicum*. *Clostridium difficile* was isolated from 3 (3.0%) of the 100 ground meat samples. All three of the positive samples were mixed beef and pork meat and came from different retailers. Two of the isolates were found to be ribotype AI-57 (Austrian Isolate-57), negative for the toxin A gene, and negative for the toxin B gene. One of these isolates was purchased from a butcher shop and the other from a grocery store so it is unlikely that the isolates came from a similar source. The third isolate was found to be ribotype 053, positive for the toxin A gene, and positive for the toxin B gene. *Clostridium*

difficile was not isolated from any of the bactofugate raw milk samples. The spores recovered from the bactofugates were from aerobic spore formers and other clostridia species. *Clostridium difficile* has not previously been isolated from raw milk samples; however, other clostridia species have been found in raw milk (137). The bactofugates were chosen to sample raw milk because these samples should contain an increased number of bacterial spores (should they be present) and this would aid isolation methods with low detection limits. *Clostridium difficile* may not have been found in these samples, because concentration levels were lower than the detection limits of the isolation procedure or else the raw milk in the bactofugates may not have been a suitable environment for *C. difficile* spores. This study found a much lower prevalence of *C. difficile* in retail meat than the studies performed in the United States and Canada (48, 50, 56). However, the 3.0% prevalence found in this study is higher than in a previous study conducted in Austria that did not detect *C. difficile* in any of the 84 retail meat samples tested (123). The enrichment technique used in this report is similar to the one used by Rodriguez-Palacios et. al. (2007, 2009) and, therefore, could be expected to yield a similar prevalence. It is also interesting that the authors did not detect *C. difficile* in any of the ground beef samples. Previous studies have found a higher prevalence of *C. difficile* in ground beef than in other retail meats (48, 50, 56, 134). The reason for the lower prevalence and differences in detection in ground beef may be due to geographic differences or in the ground meat batch sizes processed in North America versus Europe. One of the problems with this study is the procedure they used to select an isolation technique. A known strain of *C. difficile* was inoculated into ground meat and raw milk

samples. These samples had not been previously tested for *C. difficile* so it is not known if they already contained *C. difficile*. The same sample of meat was used for all 4 isolation techniques; however, *C. difficile* was not evenly distributed throughout the samples so one of the isolation techniques may have used a sample containing spores whereas another may not. It is difficult to compare culture techniques and determine detection limits when there is no gold standard available to determine if a sample is truly negative for *C. difficile*.

The majority of the studies evaluating the presence of *C. difficile* in meat have been qualitative and focused on using enrichment methods to culture the bacteria from samples. Enrichment methods can only test for the presence or absence of the bacteria and quantitative data on how many bacteria or spores are present in the sample cannot be calculated. A study in Canada evaluated the prevalence of *C. difficile* in retail meat samples using both qualitative and quantitative methods in order to estimate the levels of contamination (103). A total of 230 samples were collected from British Columbia, Saskatchewan, Ontario, and Quebec from August to November as part of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) in 2008. One hundred fifteen ground beef and 115 ground pork samples were purchased from retail stores in 17 randomly selected census divisions in each province. Qualitative methods involved a 48-hour enrichment process followed by an alcohol-shock treatment and then direct plating onto selective agar. Quantitative methods involved setting up serial 10-fold dilutions that were plated onto selective agar. *Clostridium difficile* was isolated from 28 (12%) of the 230 retail meat samples including 14 (12%) ground beef and 14

(12%) ground pork samples. Among the isolates from ground beef, 10 were positive only by the enrichment method, 2 were positive by both the enrichment method and direct plating, and 2 were positive only by direct plating. The same distribution among culture methods was seen for the 14 isolates from ground pork. The number of spores in the positive isolates from ground beef that were identified from direct plating was estimated at 20 spores per gram for two of the samples and 120 and 240 spores per gram for the other two samples. For the isolates from ground pork, as a result of direct plating, three of the samples harbored 20 spores per gram and the other sample had 60 spores per gram. Analysis using the kappa statistic found a low level of agreement (0.22) between the two methods. No geographic association was found between the prevalence of *C. difficile* and the different provinces. The majority of the isolates were found to be ribotype 078, toxinotype V which is the strain commonly found in food animals. This study is important, because it shows that the level of *C. difficile* contamination in retail meat is low and often is only detected using an enrichment process. The enrichment process used in this study had a detection limit of less than 10 spores per gram of sample. Most of the positive samples that were identified using the direct plating method also had low levels of contamination. Interestingly, two of the ground beef samples and two of the ground pork samples were identified only using the direct plating method and this may be explained by an uneven distribution of spores in the sample.

2.5 Asymptomatic carriers and Clostridium difficile *in human wastewater*

The majority of *C. difficile* studies in human populations have focused on clinical individuals in hospitals and nursing homes. A limited amount of research has been conducted on the presence of *C. difficile* in asymptomatic individuals; and only one study has been conducted to evaluate the presence of *C. difficile* in human wastewater samples (106). There have been several studies evaluating the role of asymptomatic carriers in the spread of *C. difficile* (105, 138, 139), and one study investigated the different strains found among asymptomatic individuals versus those with symptoms of diarrhea (104). The majority of the studies have evaluated asymptomatic carriers in hospitals and although there is scarce data on the percentage among the general population, it has been estimated that 1-3% of healthy adults are asymptomatic carriers of *C. difficile* (2). Typing of isolates arising from asymptomatic carriers may provide valuable information on the potential sources and routes of transmission for *C. difficile* in both hospital and community settings. Designing studies to evaluate *C. difficile* in the community is difficult and using human wastewater samples may be a valuable tool to avoid difficulties in compliance and access while protecting anonymity. The finding of *C. difficile* in biosolids provides support that human wastewater samples may be used to study the bacterium in human populations; however, more studies need to be conducted to assess whether wastewater samples provide reliable information about *C. difficile* in the human population.

A prospective study was performed in a long-term care facility to determine the prevalence of asymptomatic carriage of *C. difficile* in 2006 (105). Stool samples and

rectal swabs were collected from 73 male patients in 2 adjacent wards along with information on demographics, illnesses, fecal incontinence, and antibiotic use. Skin and environmental samples from the patients and their rooms were also cultured for *C. difficile*. Patients without CDAD during the initial survey were monitored for the subsequent six months for development of CDAD, admission to an acute care facility, or death. The patients who were initially classified as asymptomatic carriers and remained in the long-term care facility had stool samples collected 1 to 3 months after the initial sample to determine if carriage persisted. During the initial survey, 5 (6.8%) of the 73 patients had CDAD and 35 (47.9%) were asymptomatic carriers. Twelve of the 35 asymptomatic carriers had follow-up stool samples collected and 10 of these individuals had a positive fecal culture. Asymptomatic carriers were found to be more likely than non-carriers to have had previous CDAD, exposure to antibiotics in the previous 3 months, and previous exposure to fluoroquinolones. Seven of the asymptomatic carriers developed CDAD during the 6 month follow-up period. Asymptomatic carriers had significantly ($p<0.05$) higher rates of skin and environmental contamination than non-carriers; however, asymptomatic carriers had significantly ($p<0.05$) lower rates of contamination than patients with CDAD. This study provides evidence that asymptomatic carriers may contribute to transmission of *C. difficile* in health care facilities through contamination of environmental surfaces. We do not know if any of the asymptomatic carriers were released from the long-term health care facility, but some of these individuals may have returned home and had the opportunity to spread *C. difficile* in the community. The strains detected among the individuals with CDAD and

the asymptomatic carriers were not determined so no comparisons could be made about the potential spread from one population to the other or to the environment.

Clostridium difficile strains isolated from asymptomatic infants, asymptomatic children, and adult patients with diarrhea were compared using PFGE to determine the significance of transmission from asymptomatic infants and children to adults (104). A total of 446 fecal samples were collected from patients at Siriraj Hospital in Bangkok, Thailand from October 1998 to April 1999. Samples originated from 235 asymptomatic infants (<12 months old), 76 asymptomatic children (1-11 yrs old), and 132 adult patients with symptoms of diarrhea. *Clostridium difficile* was detected in 28 (11.9%) of the asymptomatic infants, 16 (21.1%) of the asymptomatic children, and 33 (25%) of the adult patients. PFGE analysis demonstrated that the majority of the toxigenic isolates from symptomatic patients had the same pattern, whereas there was a much more diverse set of patterns found among the asymptomatic infants and children. It is also interesting to note that *C. difficile* was detected in a larger percentage of asymptomatic children than infants. *Clostridium difficile* is found in the gut flora of healthy infants, but rarely causes disease; and, as an infant matures, *C. difficile* is replaced by other bacteria (commensals) found in the normal gut flora of healthy adults (140). There is evidence that the lack of toxin binding receptors and the immature nature of the gut flora in infants contribute to protection against CDAD (3). However, asymptomatic infant carriers could also serve as an important route of transmission to adults. This article provides evidence that the *C. difficile* strains found in asymptomatic infants and children are different than the strains causing disease in adults. Asymptomatic infants and children may not be an important

source of *C. difficile* in the community. However, there could also be different risk factors for clinical and community-acquired cases; and these risk factors select for certain strains of *C. difficile*.

The majority of studies on asymptomatic carriers of *C. difficile* has been conducted in health care facilities and does not address the concern of community-acquired cases. Human wastewater samples could serve as a valuable tool to investigate *C. difficile* in the community; however, there are limited data on the detection levels of the bacterium in these samples and their relevance to the human population that gave rise to the samples. The level of human pathogens (including *C. difficile*) in biosolids from wastewater facilities were evaluated in a paper published in 2009 (106). Biosolids are domestic sewer sludges that have been treated through mesophilic anaerobic digestion (MAD), temperature-phased anaerobic digestion (TPAD), or composting after anaerobic digestion (COM) to reduce pathogens; these are then used for agricultural field applications. There has been concern that residents close to farms that apply biosolids to agricultural fields may be exposed to hazardous pathogens through the soil or air. Biosolids are classified as class A (pathogen-free) or class B (contain pathogens), depending on the content of indicator organisms (bacterial and viral) or technologies used for treatment. Samples were collected from 29 wastewater facilities where the biosolids were primarily used for agricultural application. Real-time PCR was used to estimate the level of human adenovirus species, *Legionella pneumnphila*, *Staphylococcus aureus*, and *Clostridium difficile*. *Staphylococcus aureus* and *C. difficile* were included in the study, because they are human pathogens found in the environment

that can survive under extreme circumstances. *Clostridium difficile* was detected in 38% of the class A samples and 25% of the class B samples. Although *C. difficile* was found more frequently in the class A samples, the concentrations in the class A samples were significantly ($p<0.05$) lower than in the class B samples. This study is important, because it is the first and only to report the detection of *C. difficile* in biosolids from wastewater facilities. However, it is important to note that biosolids have undergone treatment procedures to remove harmful pathogens; therefore, the types and quantities of pathogens found in these samples may have little relevance to the human population from which they originate. In addition, the biosolids are a more concentrated biomass arising from wastewater, and likely have aged and changed considerably in their makeup since the fecal matter that originally was voided from the human host.

2.6 Summary

A number of studies have been conducted to estimate the incidence of CA-CDAD and also the risk factors associated with these human cases. Many of the risk factors for community-acquired cases are the same as for hospital acquired cases, such as antibiotic use and a compromised immune system. However, there have been human cases that have no history of antibiotic use and do not have any other risk factors that are normally associated with CDAD. Further research is being conducted to identify the possible sources of infection or other causes of these cases. Currently, the most widely recognized and well-published risk factors being investigated are the use of gastric-acid suppressants, and exposure to food animals or consumption of retail meats. The finding of *C. difficile* in food animals also raises concern about potential occupational exposures.

There are currently no published studies that evaluate the association between *C. difficile* infection and occupational exposure to food animals; however, occupational exposure to *C. difficile* has been documented in health care workers (124). Our study evaluated *C. difficile* in a closed population with both human and swine hosts. Within this closed population there existed a group cohort of individuals who worked with swine and a group cohort of individuals who did not work with swine. These healthy asymptomatic individuals were housed separately from one another and, therefore, we were able to estimate the aggregate-level prevalence of *C. difficile* in human wastewater arising from the occupational group cohorts in the human population. *C. difficile* has been detected in biosolids from wastewater facilities during a single study (106); however, biosolids have been treated to remove pathogens and may not serve as a good source to study *C. difficile* in human populations. Relatively freshly voided feces in human wastewater samples originating from manholes draining lavatories (and not yet subjected to treatment) therefore, may provide more accurate information concerning the bacterium in the human population. The majority of studies in human populations have focused on clinical disease in health care facilities and there are limited studies on asymptomatic populations (104, 105). Our human wastewater samples originated from asymptomatic populations in a community setting and may be more indicative of the prevalence of *C. difficile* among healthy adults than previously published studies.

There have been numerous studies that have evaluated the prevalence of *C. difficile* in swine and compared those isolates to clinical human isolates. One of the problems with previous swine studies is that these studies have focused on the

prevalence of *C. difficile* in piglets. It is well documented that *C. difficile* is found in piglets and can cause disease in piglets. However, for *C. difficile* to be evaluated as a potentially greater food safety risk, the bacterium needs to be studied in slaughter age swine. In our study we observed the prevalence of *C. difficile* in different production groups of swine including pigs in the farrowing barn, nursery barn, breeding barn, and grower/finisher barn.

Another problem with the studies that have compared swine isolates to human isolates is that these isolates have been from separate populations, often separated by vast geographic distances. Many of these studies have shown similarities between the human and swine isolates, but this does not prove that transmission is occurring. The strains in the two populations may have come from a common source. In our study, the swine and human populations were part of the same closed population within the same geographical region. We can therefore make more meaningful comparisons between the two populations.

3. MATERIALS AND METHODS

3.1 Study population

Swine composite fecal samples and human composite wastewater samples were collected from a closed, vertically integrated population in the state of Texas. The population consisted of 12 units in different geographical locations that contained both a human and swine population and an additional swine slaughter plant facility. The human population contained occupational group cohorts of swine workers and non-workers and all individuals had equal opportunity to consume the pork produced within the system. The swine population flowed vertically from the farrowing barn to the grower/finisher slabs and all swine were slaughtered and consumed within the system.

3.1.1 Swine population

There were 12 swine units in different geographical locations that housed a total of approximately 26,000 to 28,000 swine during a given month. The units included five farrow-to-finish units, six grower-finisher units, and one purebred boar quarantine unit. The swine traveled vertically through the system from the farrowing barns to the hot nursery to the cold nursery and finally to the grower/finisher barn (or to the barns in other grower/finisher units). Production groups were categorized as breeding boars and sows, farrowing barn pigs (lactating sows, suckling piglets), weaned nursery piglets, and grower and finisher pigs. All of the swine were raised within the operation and remained in the system until slaughter. Swine were sent to a central slaughter plant where pork products were processed for consumption by the human population. There

were some purebred boars that were occasionally purchased and entered the system for breeding purposes. These boars were housed in a single quarantine unit for approximately 4 weeks prior to entering the system.

3.1.2 Human population

The targeted human population was comprised of approximately 39,000 male individuals who were housed in 13 units in different geographical locations. Individuals in the population were categorized as either swine workers or non-swine workers based on their occupational exposure to the swine population. There were 12 units with swine populations and 1 unit with a slaughter plant facility, where both workers and non-workers were housed. The swine workers and swine non-workers were housed separately. All individuals had the opportunity to consume the pork products processed in the system.

3.2 Sampling

3.2.1 Swine fecal sampling

Swine fecal samples were collected monthly at each of the 12 units from February 2004 to January 2007. Composite fecal samples were each comprised of fecal pats from multiple pens containing feces from about ten asymptomatic, clinically healthy swine. Several representative composite fecal samples were collected for each of the production groups mentioned above. Pre-lagoon effluent samples were collected from specific locations that drained from the sampled pens. The composite fecal and pre-lagoon effluent samples were collected into sterile 50-ml conical tubes (BD, Franklin Lakes, NJ). The samples were stored on ice and transported to the Food and Feed Safety

Research Unit (FFSRU) laboratory, USDA, ARS, College Station, TX. Upon arrival, the samples were stirred to ensure uniform conformity and then 4 ml was placed into a 5-ml tube containing 1 ml of sterile glycerol. The samples were sealed and vortexed to distribute the glycerol evenly throughout the sample. There were 3 replicates of 5-ml tubes produced for each original sample. Samples were stored at -80°C until further microbiological analysis was performed.

3.2.2 Human wastewater sampling

Human wastewater samples were collected monthly at each of the 13 units from February 2004 to January 2007. Typically, 3 swine-worker and 3 swine-non-worker samples were collected from each unit every month. Samples were collected from representative manholes (i.e., directly draining lavatories of the two respective occupational cohorts) into 50-ml tubes. Samples were stored on ice and shipped to the FFSRU laboratory. Upon arrival, the wastewater samples were stirred to ensure uniformity and then 4 ml was placed into a 5-ml tube containing 1 ml of sterile glycerol and stored at -80°C. There were 3 replicates of 5-ml tubes produced and stored for each original sample.

3.3 Isolation of bacterium

3.3.1 Isolation from swine

Isolation of *C. difficile* was performed utilizing an enrichment procedure, alcohol-shock treatment, and restrictive media technique similar to the methods used by Rodriquez-Palacios et al. (95). Samples containing 4 ml of sample and 1 ml of glycerol were allowed to thaw to room temperature on the laboratory bench and then transferred

into an anaerobic chamber. The tubes were stirred to ensure a uniform composition and then 1 g was weighed out and added to a 15-ml tube containing 2 ml of 96% ethanol. The samples were agitated on a rotator for 50 minutes aerobically and then centrifuged aerobically at 3,800 x *g* for 10 minutes. In an anaerobic chamber the supernatant was removed from the tubes and the sediment was suspended in 5 ml of cycloserine cefoxitin fructose broth (CCFB) (Table 1). The enriched samples were incubated for seven days anaerobically at 37°C. On the seventh day, 5 ml of 96% ethanol was added to the tubes in the anaerobic chamber. The tubes were then centrifuged aerobically at 3,800 x g for 10 minutes. In the anaerobic chamber, the supernatant was removed, the sediment was suspended in 600 µl of sterile de-ionized water, and then 200 µl of the suspended sediment was spread onto a cycloserine cefoxitin fructose agar (CCFA) plate (Anaerobe Systems, Walnut, California.). The plates were incubated anaerobically at 37°C and checked daily for 5 days for growth. *C. difficile* were identified as yellow, broken glass appearing colonies on the plate. A single suspect colony from each positive plate was streaked onto an anaerobic brucella plate (Anaerobe Systems) and incubated for 48 hours anaerobically. After 48 hours, the suspect isolate was confirmed as *C. difficile* using API (Rapid ID 32A, bio-Merieux, Durham, NC) per the manufacturer's instructions.

Table 1. Recipe and instructions for cycloserine cefoxitin fructose broth (CCFB).

Add the following ingredients to 500 ml of de-ionized water.

Ingredient	Amount (grams)
Proteose peptone (Remel, Lenexa, KS)	20
Disodium hydrogen phosphate (Na_2HPO_4) (Fisher Scientific, Fair Lawn, NJ)	2.5
Potassium dihydrogen phosphate (KH_2PO_4) (Fisher Scientific, Fair Lawn, NJ)	0.5
Magnesium sulphate (Fisher Scientific, Fair Lawn, NJ)	0.05
Sodium chloride (VWR International, West Chester, PA)	1
Fructose (Fisher Scientific, Fair Lawn, NJ)	3

Stir ingredients together and then autoclave for 20 minutes.
Allow autoclaved broth to cool to room temperature before adding selective supplement.

Clostridium difficile Selective Supplement SR0096E (Oxoid Ltd., Basingstoke, Hampshire, UK)

Antibiotic	Amount (milligrams)
D-cycloserine	125
Cefoxitin	4

Aseptically add 2 ml of sterile water to the selective supplement and mix well.
Add the selective supplement to the cooled broth and mix well.

3.3.2 Isolation from wastewater

Isolation of *C. difficile* from the wastewater samples was performed in a similar manner as for the swine samples except for one modification during the plating step. On the seventh day of enrichment, 96% ethanol was added to the samples anaerobically and then the samples were centrifuged aerobically in the same manner as the swine samples. The difference for the wastewater samples as compared to the swine samples, was that after the supernatant was removed anaerobically, the sediment was suspended in 200 µl

(instead of the 600 µl used for the swine samples) of sterile de-ionized water, and then 200 µl of the suspended sediment was spread onto a CCFA plate (Anaerobe Systems). The plates were incubated anaerobically at 37°C and checked daily for 5 days for growth. *Clostridium difficile* were identified as yellow, broken glass appearing colonies on the plate. A single suspect colony from each positive plate was streaked onto an anaerobic brucella plate (Anaerobe Systems) and incubated anaerobically for 48 hours. After 48 hours, the suspect isolate was confirmed to be *C. difficile* using API (Rapid ID 32A) per the manufacturer's instructions.

3.4 Molecular analysis

3.4.1 DNA preparation

Isolates confirmed to be *C. difficile* by API were inoculated in Viande-Levure broth (VL) (Table 2) and incubated anaerobically for 24 hours at 37°C. The positive cultures were kept at 4°C until DNA extraction was performed. DNA extraction was performed using a QIAamp DNA Mini Kit (QIAGEN Sciences, Valencia, CA). Procedures followed the manufacturer's instructions for isolation of genomic DNA from Gram-positive bacteria. A bacterial pellet was obtained by centrifugation of the VL culture broth for 10 minutes at 3,800 x *g*. The bacterial pellet was suspended in 180 µl of lysozyme (20 mg/ml) (Amresco, Solon, OH) and incubated for 30 min at 37°C. After 30 minutes, 20 µl of proteinase K and 200 µl of Buffer AL (both provided in the kit) were added to the suspension and vortexed. The samples were incubated at 56°C for 30 minutes and then for an additional 15 minutes at 95°C. The samples were then briefly centrifuged to remove liquid from the sides of the tubes and 200 µl of Buffer AL was

added. The samples were vortexed for 15 seconds and incubated at 70°C for 10 minutes. After the incubation period, the samples were briefly centrifuged and 200 µl of 96% ethanol was added. The samples were vortexed for 15 seconds and then briefly centrifuged. The samples were then pipetted into the QIAamp Mini spin column and centrifuged at 6,000 x *g* for 1 minute. The QIAamp Mini spin column was then placed in a clean 2-ml collection tube and the old collection tube containing the filtrate was discarded. To the spin column, 500 µl of Buffer AW1 (provided) was added and the samples were centrifuged at 6,000 x *g* for 1 minute. Once again, the spin column was placed in a clean 2-ml collection tube and the old tube containing the filtrate was discarded. To the spin column, 500 µl of Buffer AW2 (provided) was added and the samples were centrifuged at 20,000 x *g* for 3 minutes. The spin columns were placed in a clean 2-ml collection tube and the old tube containing the filtrate was discarded. The samples were centrifuged at 20,000 x *g* for an additional minute. The spin columns were placed in a clean 1.5-ml microcentrifuge tube and the collection tube containing the filtrate was discarded. To the spin column, 200 µl of Buffer AE (provided) was added. The samples were incubated at room temperature for 1 minute and then centrifuged at 6,000 x *g* for 1 minute. The spin columns were discarded and the 1.5-ml microcentrifuge tubes containing the isolated DNA were stored at 4°C for further analysis. The concentration of the isolated DNA was measured using a spectrophotometer and all samples were diluted to 25 ng/µl using sterile Tris-EDTA (TE) buffer (10 ml of 1M Tris (GBiosciences, St. Louis, Mo) and 2 ml of 0.5M EDTA (Amresco, Solon, OH)).

Table 2. Recipe and ingredients for Viande-Levure broth.

Add the following ingredients to 500 ml of de-ionized water.

Ingredient	Amount (grams)
Tryptose (Becton, Dickinson, and Company, Sparks, MD)	5
Yeast Extract (Becton, Dickinson, and Company, Sparks, MD)	2.5
Sodium Chloride (NaCl) (VWR International, West Chester, PA)	2.5
Beef Extract (Becton, Dickinson, and Company, Sparks, MD)	1.2
Cysteine- HCl (Thermo Scientific, Rockford, IL)	0.3
Dextrose (Becton, Dickinson, and Company, Sparks, MD)	1.25

Stir ingredients together and dispense 9 ml into glass tubes. Place tubes in the anaerobic chamber overnight to reduce. Place stoppers and screwcaps on tubes and autoclave for 30 minutes.

3.4.2 Polymerase chain reactions

Polymerase chain reactions (PCR) were used to test for the presence of the toxin A and toxin B genes, the *tcdC* gene deletion, and the binary toxin gene. Three different PCR reactions were conducted using a thermocycler (Px2 Thermal Cycler, Thermo Electron Corporation, Waltham, MA) and the PCR primers used for amplification of the 4 target genes are in Table 3. Later, genomic characteristics of the isolates were compared using PCR toxinotyping and the PCR procedures followed slightly modified protocols as those utilized by the Centers for Disease Control and Prevention (CDC), Atlanta, GA (29, 141, 142).

Table 3. PCR primers used for amplification of *tcdC* gene, binary toxin gene, toxin A gene, and toxin B gene.

Target gene	Primer name	Primer sequence (5'-3')
tcdC gene	Tim2	GCA CCT CAT CAC CAT CTT CAA [1]
	Struppi2	TGA AGA CCA TGA GGA GGT CAT [1]
Binary toxin gene	Cdtpos	CTT AAT GCA AGT AAA TAC TGA G [2]
	Cdtrev	AAC GGA TCT CTT GCT TCA GTC [2]
Toxin A gene	A3C	TAT TGA TAG CAC CTG ATT TAT ATA CAA G [1]
	A4N	TTA TCA AAC ATA TAT TTT AGC CAT ATA TC [1]
Toxin B gene	B1C	AGA AAA TTT TAT GAG TTT AGT TAA TAG AAA [1]
	B2N	CAG ATA ATG TAG GAA GTA AGT CTATAG [1]

[1] Spigaglia P, Mastrantonia P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (tcdC) among Clostridium difficile clinical isolates. J Clin Microbiol. 2002;40(9):3470-5.

[2] Goncalves C, Decre D, Barbut F, Burghoffer B, Petit JC. Prevalence and characterization of a binary toxin (actin-specific ADP-ribosyltransferase) from Clostridium difficile. J Clin Microbiol. 2004; 42(5):1933-9.

3.4.2.1 Polymerase chain reaction for detection of the *tcdC* gene

The master mix for the PCR reaction for the *tcdC* gene consisted of 12.5 µl Jumpstart RedTaq ReadyMix (Sigma Chemical Company, St. Louis, MO), 1.0 µl Bovine Serum Albumin (10X) (BSA) (New England BioLabs, Ipswich, MA), 4.5 µl DNA-free water (Mo BIO Laboratories, Inc., Carlsbad, CA), 2.0 µl of Tim2 primer (50 µM) (Integrated DNA Technologies, Inc.(IDT), Coralville, IA), and 2.0 µl Struppi2 primer (50 µM) (IDT) for each sample. The 22 µl of master mix was added to 3.0 µl of *C. difficile* DNA template. The samples were kept on ice during the procedure and once master mix was added to the template of all the samples, the samples were gently centrifuged for a few seconds and then placed in the thermocycler. The thermocycler protocol began with a 5-minute denaturation cycle at 95ºC. The next 40 cycles consisted

of a 1-minute denaturation step at 95ºC, followed by 1-minute annealing step at 52ºC, and finally a 1-minute extension step at 72ºC. Once the amplification process was complete the samples were held at 4ºC in the thermocycler until they could be transferred to the freezer. Amplification products were run on a 2% agarose gel with three different controls. The agarose gel was made by mixing 2 grams of agarose powder (Molecular Biology Grade, Fisher Scientific) with 100 ml of 0.5 M solution of Tris-Borate-EDTA (pH 8) (TBE) (Fisher Scientific) buffer in a small glass flask. The agarose mixture was heated in the microwave until the agarose was completely in solution. The agarose was allowed to cool on the laboratory bench until it was cool enough to handle (about 5 minutes) and then 2 µl of ethidium bromide (1% solution) (Fisher Scientific) was added to the agarose. The agarose was slowly poured into the gel mold containing two, 20-well gel combs and allowed to cool for 20 to 30 minutes. The gel combs and sides of the mold were removed and enough 0.5 M TBE solution was poured into the gel apparatus until the gel was completely submerged by at least 1mm. A 100-bp ladder (10X) (Lonza, Rockland, ME) was added to the first well of each gel, followed by four controls. Samples were loaded into the remaining wells by adding 5 µl of loading dye (6X) (Lonza) to each 25 µl sample, mixing the sample well with the pipette, and then loading 5 µl of the sample/loading dye into a well. The four controls included a negative control that did not contain *C. difficile* DNA template, a wild type strain that did not contain a deletion in the *tcd* gene, a sample with an 18-bp deletion in the *tcd* gene, and a sample with a 39-bp deletion in the *tcd* gene. The gel was run at 140 volts for 60 minutes and then imaged.

3.4.2.2 Polymerase chain reaction for the binary toxin gene

The master mix for the binary toxin gene PCR was prepared by combining 12.5 µl Jumpstart RedTaq ReadyMix, 1.0 µl BSA (10X), 3.5 µl DNA-free water, 2.5 µl cdtpos primer (50 µM) (IDT), and 2.5 µl cdtrev primer (50 µM) (IDT). The 22 µl of master mix was added to 3 µl of *C. difficile* DNA template. The samples were kept on ice during the procedure and once master mix was added to the template for all of the samples, the samples were gently centrifuged for a few seconds and then placed in the thermocycler. The thermocycler protocol began with a 5-minute denaturation cycle at 95ºC. The next 35 cycles consisted of a 45-second denaturation step at 95ºC, followed by a 1-minute annealing step at 49ºC, and finally a 1-minute and 20-second extension step at 72ºC. The samples were held at 4ºC in the thermocycler until they could be transferred to the freezer. Amplification products were run on a 1.5% agarose gel at 140 volts for 60 minutes and then imaged. The agarose gel was prepared using the same steps mentioned above using 1.5 grams of agarose and 100 ml of 0.5 M TBE solution. The samples were run with a negative control that did not contain *C. difficile* DNA template and also a positive control that was known to contain the binary toxin gene.

3.4.2.3 Polymerase chain reaction for detection of the toxin A and toxin B genes

The PCR for the toxin A and toxin B genes were run using the same master mix components and thermocycler protocol, except for the unique primers for each gene. The master mix for the toxin A PCR reaction consisted of 2.5 µl Accuprime Buffer II (10X) (Invitrogen), 0.5 µl Accuprime Taq (Invitrogen), 14.5 µl DNA-free water, 2.5 µl

A3C primer (5µM) (IDT), and 2.5 µl A4N primer (5µM) (IDT). The master mix for the toxin B PCR reaction used the same amounts of Accuprime Buffer II, Accuprime Taq, and DNA-free water with 2.5 µl B1C primer (5µM) (IDT) and 2.5 µl B2N primer (5µM) (IDT). The 22.5 µl of master mix for the toxin A gene was added to 2.5 µl of *C. difficile* DNA template and 22.5 µl of master mix for the toxin B gene was added to a separate tube with 2.5 µl of *C. difficile* DNA template. The samples were kept on ice during the procedure and once master mix was added to the template for all of the samples, the samples were gently centrifuged for a few seconds and then placed in the thermocycler. The toxin A and B tubes were run together in the thermocycler with an initial denaturation cycle for 5-minutes at 95ºC. The next 35 cycles included a 30-second denaturation step at 95ºC, followed by 30-second annealing step at 47ºC, and finally a 3-minute extension step at 68ºC. Samples were held in the thermocycler at 4ºC until they could be transferred to the freezer. Amplification products were run on a 1.5% agarose gel at 140 volts for 60 minutes and then imaged. The agarose gel was prepared using the same instructions mentioned above. The samples were run with a negative control that did not contain *C. difficile* DNA template and also a positive control that was known to contain the toxin A and toxin B genes.

3.4.2.4 Polymerase chain reaction for toxinotyping

The toxinotyping PCR consisted of three different reactions, run in three separate tubes, that were run in the thermocycler at the same time. The three reactions used different restriction enzymes; *Eco*RI, *Hinc*II, and *Acc*I. The DNA templates for the reactions were the amplification products from the toxin A and toxin B gene PCR. The

first reaction included 11.75 µl DNA-free water, 2.0 µl Buffer 3 (10X) (New England BioLabs), and 0.25 µl *Eco*RI enzyme (5U) (New England BioLabs) added to 6.0 µl of the toxin A amplification product. The second reaction included 9.5 µl DNA-free water, 2.0 µl Buffer 3 (10X), 2.0 µl BSA (10X), and 0.5 µl *Hinc*II enzyme (5U) (New England BioLabs) added to 6.0 µl of the toxin B amplification product. The third reaction included 11.5 µl DNA-free water, 2.0 µl Buffer 4 (10X) (New England BioLabs), and 0.5 µl *Acc*I enzyme (5U) (New England BioLabs) added to 6.0 µl of the toxin B amplification product. The tubes were kept on ice during the process and then all the tubes were gently centrifuged for a few seconds and placed in the thermocycler. The samples were held at 37ºC in the thermocycler overnight and then transferred to the freezer. The reaction products were run on a 1.5% agarose gel at 140 volts for 60 minutes. The agarose gel was prepared as previously described.

3.4.3 Sequencing of the tcdC *gene deletion*

A representative sample of *tcdC* genes from the different toxinotypes found among the *C. difficile* isolates and three control samples was sent to the DNA Technologies Core Laboratory at the College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX. The genes were sequenced to determine the presence and size of the deletions in the *tcdC* gene. The three controls submitted included a wild strain with no deletion, a toxinotype III strain with an 18-bp deletion, and a toxinotype V strain with a 39-bp deletion. We provided the amplification products from the PCR for the *tcdC* gene as well as the Tim2 primer. Sequence products were separated and detected on an ABI 3130xl Genetic Analyzer by the DNA

Technologies Core Laboratory. Once the results were provided by the DNA Technologies Core Laboratory; the Basic Local Alignment Search Tool (BLAST) was used to confirm that the products were *C. difficile* and to compare with other strains of *C. difficile* in the database. Nucleotide sequences of the representative samples were visually compared to the three controls to determine the size of the deletion.

3.4.4 Pulsed-field gel electrophoresis

Clostridium difficile isolates were further compared using pulsed-field gel electrophoresis (PFGE). The PFGE procedure followed modified techniques according to a 7-day protocol utilized by the CDC (Angela Thompson, CDD, personal communication). On the first day, an isolate was inoculated onto an anaerobic brucella agar plate (Anaerobe Systems) and incubated anaerobically for 48 hours at 37°C. On day two, 2 ml of protein yeast glucose (PYG) broth (PathCon, Norcross, GA) in a 15-ml falcon tube for each sample was placed in the anaerobic chamber with a loosened cap and left overnight to reduce. The following day, the PYG broth was inoculated with a single large *C. difficile* colony and after the culture incubated for 7 hours, it was used to make PFGE agarose plugs. Agarose plugs were made by combining a bacterial pellet from 750 µl of the PYG broth culture, 300 µl Gram-Positive Lysis Buffer, and 350 µl of agar (0.36 g SeaKem Gold agarose (Lonza) in 20 ml Tris-EDTA (TE) buffer (10 ml of 1M Tris (GBiosciences, St. Louis, MO) and 2 ml of 0.5M EDTA (Amresco); dilute to 1000 ml with RO (reverse osmosis) water)). The Gram-positive lysis buffer was prepared ahead of time by mixing 10.8 ml Tris-HCl (1 M, pH 8.0) (Gibco, Carlsbad, CA), 360 ml NaCl (5 M) (Amresco), 360 ml EDTA (0.5 M, pH 8.0) (Amresco), 9 g Brij-

58 (Polyoxyethylene 20 Cetyl Ether) (Sigma), 3.6 g sodium deoxycholate (Sigma), and 9 g sodium lauryl sarcosine (Sigma) in a beaker. The solution was thoroughly mixed with a stir bar, poured into a 2000-ml cylinder, and allowed to settle overnight. The next day, enough RO water was added to bring the volume of the buffer up to 1800 ml. The buffer was filter-sterilized using a 0.22-µm disposable filter and kept at room temperature until ready to use. The plugs were left in lysis buffer (for each plug; 3 ml Gram-Positive Lysis Buffer; 60 µl RNase (20 mg/ml) (Amresco); 15 mg lysozyme (Amresco)) overnight. The fourth day involved pouring out the lysis buffer and adding proteinase K and Sodium lauryl sulfate (for each plug; 3 ml EDTA (0.5M, pH 8.0) (Sigma); 30 mg Sodium lauryl sulfate (Fisher Scientific); 3 mg proteinase K (20 mg/ml) (Amresco)) to the plugs to dissolve cell membranes and inactivate the proteins. The proteinase K was used to protect the DNA from degradation during the purification. The plugs were washed 4 times on day 5 with TE buffer for 30 minutes to remove the SDS and proteinase K on day 5. On the sixth day, the plugs were restricted with 3 µl *Sma*I (New England Biolabs Inc.) in 150 µl Buffer 4 (10X) (New England BioLabs) and 2 µl BSA (10X) and incubated at room temperature for at least 4 hours. Enough *Salmonella Braenderup* reference plugs were restricted to place one in every five lanes. The *Salmonella* plugs were prepared according the instructions available on the CDC website for PulseNet (http://www.cdc.gov/pulsenet/protocols.htm). The *Salmonella* plugs were restricted with 5 µl *Xba*I (50 units) (New England Biolabs, Inc.) in 175 µl RO water and 20 µl of Buffer 2 (New England Biolabs, Inc.) and incubated in a 37ºC water bath for at least 4 hours. A 2000-ml TBE buffer solution with thiourea (200 µM) (Sigma) was used

to make the gel and the remaining buffer was added to the gel chamber. The plugs were loaded into a gel prepared by combining 1.5 g SeaKem Gold Agarose and 150 ml of TBE. The gel was run on a Bio-Rad CHEF Mapper system (Bio-Rad, Hercules, CA) with the following settings: two state, gradient of 6, run time of 18 hours, included angle of 120, initial switch time of 5 seconds; final switch time of 40 seconds; ramping factor of linear. The gel was stained with 25 µl of ethidium bromide (1% solution) in 500 ml of RO water for 15 minutes on a rotator, de-stained in 1000-ml RO water for 60 minutes, and imaged. Images were analyzed using BioNumerics software (Applied Maths, Austin, TX).

Once all PFGE patterns were determined, a final master gel was run containing one representative isolate for each unique pattern. The image from this master gel was imported into BioNumerics software for dendrogram analysis. Banding patterns were standardized using the *Salmonella Braenderup* placed every 6 lanes on the gel. In the comparison window we performed a cluster analysis on the master gel. The cluster analysis was based on a matrix of similarities between the isolate patterns and a subsequent algorithm for calculating the dendrogram that represented the clusters among the isolates (BioNumerics manual, version 5.1). The resulting dendrogram showed the average percentage similarity between the individual isolate patterns. Along with the dendrogram, a similarity matrix was produced. The similarity matrix contains the detailed comparison data for of all the isolates; and the comparison data between any two isolate patterns can be easily viewed. We chose not to include a cluster cutoff value

at a given similarity value to differentiate between relevant and non-relevant clusters; instead we chose to classify all of our isolate patterns as relevant.

3.5 Antibiotic sensitivity testing

Antibiotic sensitivity testing was performed using Etest® (AB Biodisk™ North America, Inc., Piscatway, NJ). Eleven different antibiotics were tested including, ampicillin, chloramphenicol, tetracycline, amoxicillin/clavulanic acid, imipenem, cefoxitin, metronidazole, ciprofloxacin, clindamycin, piperacillin/tazobactam, and vancomycin. *Clostridium difficile* isolates were grown anaerobically for 48 hours on anaerobic brucella plates (Anaerobe Systems) at 37ºC. Enough culture from the brucella plate was added to 1 ml of VL broth to bring the broth to a McFarland standard of 0.5. Six brucella agar plates were streaked in three different directions with the VL broth culture using a sterile cotton swab per the instructions of the manufacturer (AB Biodisk™ North America, Inc.). Two different antibiotic strips were placed on the plate in opposite directions and spaced about 2cm apart. Plates were incubated anaerobically at 37°C and read as per the manufacturer's instructions at 24 hours and then again at 48 hours. Results were reported at 48 hours and interpreted according to the breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI) (USA, 2009) (Table 4). Anaerobic breakpoints for Etests were not provided for vancomycin or ciprofloxacin. Vancomycin results were interpreted based on the CLSI breakpoints reported for Gram-positive aerobes and ciprofloxacin results were interpreted based on trovafloxacin CLSI breakpoints reported for Gram-positive anaerobes (Table 4). Quality

control strains *Bacterioides fragilis* (ATCC # 25285) and *B. thetaiotaomicron* (ATCC # 29741) were tested and interpreted using the recommended CLSI breakpoints.

Table 4. Interpretive MIC breakpoints for antimicrobial susceptibility data for *Clostridium difficile*.

Antimicrobial	MIC S [1]	R
Amoxicillin-clavulanic acid	≤ 4	≥ 16
Ampicillin	≤ 0.5	≥ 2
Cefoxitin	≤ 16	≥ 64
Chloramphenicol	≤ 8	≥ 32
Clindamycin	≤ 2	≥ 8
Imipenem	≤ 4	≥ 16
Metronidazole	≤ 8	≥ 32
Piperacillin-tazobactam	≤ 32	≥ 128
Tetracycline	≤ 4	≥ 16
Ciprofloxacin [2]	≤ 2	≥ 8
Vancomycin [3]	≤ 4	≥ 32

[1] Sensitive and Resistant MIC values from CLSI

[2] interpretation based on MIC for trovafloxacin

[3] interpretation based on MIC for Gram-positive aerobes

3.6 Statistical analysis

3.6.1 Descriptive statistics

Descriptive statistics on the prevalence of *C. difficile* in the swine and human populations were generated for the phenotypic characteristics using cross tabulation by year, month, season, location, and production group/group cohort. Months were collapsed into seasons as follows; winter (December, January, February), spring (March, April, May), summer (June, July, August), and fall (September, October, November). In the swine population, the production groups explored were farrowing, grower/finisher,

nursery, and breeding. In the human population, the group cohorts were swine-workers and the swine-non-workers. Genotypic characteristics were also explored using descriptive statistics both within and between the host species. The genotypic characteristics included the size of the *tcdC* gene deletion, presence or absence of the binary toxin gene, toxin A, and toxin B genes, toxinotype, and PFGE pattern. Fisher's exact test was used to examine unconditional associations both within and between host species ($p<0.05$) and exact confidence intervals were calculated (in Stata SE Release 10.1, Stata Corp., College Station, TX).

3.6.2 Multi-variable logistic regression of phenotypes

Mixed model logistic regression (xtlogit-(RE) in Stata SE Release 10.1), taking into account both fixed and random factors, was used to examine the association between risk factors and *C. difficile* presence within the host species. The random effect in the model was the geographical swine unit to account for the dependency of responses by location. The fixed factors included season, swine production group/ human occupational group cohort, as well as the interaction between season and production type/group cohort.

3.6.3 Multi-level mixed effects logistic regression of phenotypes

Multi-level mixed effects logistic regression (xtmelogit in Stata SE Release 10.1) was used to explore the association between phenotypic characteristics, both within and between host species and prevalence of *C. difficile*. The random factors in the model were unit and year to account for the dependency of responses by location and time. The fixed factors (phenotypic characteristics) included season, month, and swine production

group/ human occupational group cohort. Season, month, and production group (for the swine population), were nominal variables whereas occupational group cohort (for the human population) was a binary variable. The interactions of the significant fixed factors were also explored.

3.6.4 Analysis of antimicrobial susceptibility data

The antimicrobial susceptibility profiles for the host species were explored using Fisher's exact test and non-parametric survival analysis. The susceptibility, coded as binary, to each antibiotic was explored between and within host species using Fisher's exact test ($p<0.05$) (-tabulate- in Stata SE Release 10.1). Antimicrobial data was collapsed into binary categories by collapsing the susceptible and intermediate breakpoings into one category and then the second category included the resistant isolates. Non-parametric survival analysis was used to assess differences in the distribution of MIC values both within and between the host species (-stset- and –stsum- in Stata SE Release 10.1). Log-rank, Cox, Wilcoxon, and Tarone-Ware tests were used to test the equality of the MIC distributions both within and between host species (-sts-test in Stata SE Release 10.1). Kaplan-Meier survival curves were produced to visually compare the MIC distributions (-sts- graph in Stata SE Release 10.1). Etest MIC values of greater than 32 (for ciprofloxacin and imipenem) or greater than 256 (for amoxicillin/clavulanic acid, ampicillin, cefoxitin, chloramphenical, clindamycin, metronidazole, piperacillin/tazobactam, tetracycline, and vancomycin) were treated as right-censored observations.

4. RESULTS

4.1 Descriptive statistics

4.1.1 Swine descriptive statistics

A total of 2,936 swine samples were tested and 252 of the samples (8.6%) were culture positive for *C. difficile*. The prevalence of *C. difficile* varied across the three years from a high of 13.6% in 2006 to a low of 3.9% in 2005 (Table 5). The prevalence was significantly ($p<0.05$) different among the production groups with the highest prevalence (24.9%) found in the farrowing barn and the lowest prevalence (2.7%) found in the grower/finisher swine (Table 6). The prevalence did not differ significantly ($p=0.96$) among the seasons (Table 7). The average monthly prevalence was 8.5% and varied from a low of 5.0% in July to a high of 12.1% in September (Table 8). Across the 12 swine production units the prevalence varied from 14.6% to 0.9% (Table 9). Units one, five, six, and seven, had the highest prevalence and all four of these units were farrow-to-finish units. Unit three was also a farrow-to-finish unit, and this unit had a much lower prevalence than the other farrow-to-finish units.

Table 5. Prevalence of *Clostridium difficile* in the swine fecal samples from 2004 to 2006.

Year	Prevalence	95% Confidence Interval
2004	8.6% (86/999)	(6.9, 10.3)
2005	3.9% (39/1002)	(2.7, 5.1)
2006	13.6% (127/935)	(11.4, 15.8)
Overall	8.6% (252/2936)	(7.6, 9.6)

Table 6. Prevalence of *Clostridium difficile* across the swine production groups.

Production group	Prevalence	95% Confidence Interval
Farrowing	24.9% (175/703)	(21.7, 28.1)
Nursery	5.1% (14/276)	(2.5, 7.7)
Breeding	4.3% (26/604)	(2.7, 5.9)
Grower/finisher	2.7% (37/1353)	(1.9, 3.6)

Table 7. Seasonal prevalence of *Clostridium difficile* among swine composite fecal samples.

Season	Prevalence	95% Confidence Interval
Fall	9.0% (63/701)	(6.7, 11.1)
Winter	8.7% (61/702)	(6.6, 10.8)
Spring	8.5% (69/814)	(6.6, 10.4)
Summer	8.2% (59/719)	(6.2, 10.2)

Table 8. Monthly prevalence of *Clostridium difficile* among swine composite fecal samples.

Month	Prevalence	95% Confidence Interval
January	8.9% (46/459)	(5.5, 12.4)
February	9.3% (32/432)	(5.7, 12.9)
March	8.4% (50/445)	(4.9, 11.8)
April	8.7% (44/452)	(5.4, 12.1)
May	8.3% (67/482)	(5.1, 11.5)
June	10.7% (41/468)	(7.0, 14.3)
July	5.0% (35/399)	(2.1, 7.9)
August	8.4% (43/409)	(4.8, 12.0)
September	12.1% (49/439)	(7.9, 16.3)
October	7.6% (38/423)	(4.1, 11.0)
November	7.3% (34/438)	(4.1, 10.6)
December	7.6% (44/382)	(3.9, 11.3)

Table 9. Prevalence of *Clostridium difficile* in swine fecal samples from the 12 swine production units (ranked from highest to lowest prevalence).

Unit	Prevalence	95% Confidence Interval	Unit type
1	14.6% (59/404)	(11.3, 18.4)	farrow-to-finish
6	14.5% (57/392)	(11.2, 18.4)	farrow-to-finish
7	11.6% (47/406)	(8.6, 15.1)	farrow-to-finish
5	10.9% (49/449)	(8.2, 14.2)	farrow-to-finish
2	6.5% (8/124)	(2.8, 12.3)	grower-finisher
8	4.6% (6/130)	(1.7, 9.8)	grower-finisher
12	4.1%(8/193)	(1.8, 8.0)	grower-finisher
11	4.1%(5/122)	(1.3, 9.3)	grower-finisher
4	2.5% (3/118)	(0.5, 7.3)	grower-finisher
3	1.9% (7/360)	(0.8, 4.0)	farrow-to-finish
9	1.6% (2/124)	(0.2, 5.7)	grower-finisher
10	0.9%(1/114)	(0.02, 4.8)	grower-finisher

4.1.2 Human descriptive statistics

There were 2,292 human wastewater samples tested and 271 of the samples (11.8%) were culture positive for *C. difficile*. The prevalence of *C. difficile* varied across the three years from a low of 5.8% in 2006 to a high of 18.6% in 2005 (Table 10). There was no significant difference ($p=0.42$) in the prevalence of *C. difficile* between the swine worker and swine non-worker occupational group cohorts (Table 11). The prevalence of *C. difficile* differed significantly ($p<0.05$) between the seasons, with a higher prevalence found during the spring; this included the months of March, April, and May (Table 12). The average monthly prevalence was 11.6% and varied from an average low of 4.9% in February to a high of 22.2% in May (Table 13). Across the units, the prevalence varied from a low of 7.6% in Unit 12 to a high of 17.2% in Unit 7 (Table 14). Units 7 and 3 had the highest prevalence of *C. difficile* in the human wastewater samples and both of these units were farrow-to-finish units. However, when we further compared the prevalence of *C. difficile* in the swine samples and human wastewater samples across the units we found that units with a high prevalence in the swine samples did not necessarily have a high prevalence in the human wastewater samples (Table 15). Unit 3 had the lowest prevalence in the swine samples among the farrow-to-finish units; however, it had the second highest prevalence among the human samples. Unit 13 is the slaughter plant facility and it did not have a corresponding swine population.

Table 10. Prevalence of *Clostridium difficile* among the human wastewater samples from 2004 to 2006.

Year	Prevalence	95% Confidence Interval
2004	10% (82/818)	(8.0, 12.1)
2005	18.6% (150/805)	(15.9, 21.3)
2006	5.8% (39/669)	(4.1, 7.6)
Overall	11.8% (271/2292)	(10.5, 13.1)

Table 11. Prevalence of *Clostridium difficile* among the human occupational group cohort wastewater samples.

Cohort	Prevalence	95% Confidence Interval
Swine worker	12.0% (131/1090)	(10.1, 14.0)
Swine non-worker	11.6% (140/1202)	(9.8, 13.5)

Table 12. Seasonal prevalence of *Clostridum difficile* among the human wastewater samples.

Season	Prevalence	95% Confidence Interval
Spring	16.3% (92/565)	(13.2, 19.3)
Summer	10.8% (60/557)	(8.2, 13.4)
Winter	10.7% (61/571)	(8.1, 13.2)
Fall	9.7% (58/599)	(7.3, 12.1)

Table 13. Monthly prevalence of *Clostridium difficile* among the human wastewater samples.

Month	Prevalence	95% Confidence Interval
January	11.4% (23/202)	(7.0, 15.8)
February	4.9% (9/185)	(1.8, 8.0)
March	14.9% (29/194)	(9.9, 20.0)
April	11.3% (20/177)	(6.6, 16.0)
May	22.2% (43/194)	(16.3, 28.0)
June	6.1% (12/196)	(2.8, 9.5)
July	13.5% (24/178)	(8.4, 18.5)
August	13.1% (24/183)	(8.2, 18.0)
September	10.1% (21/208)	(6.0, 14.2)
October	10.6% (21/198)	(6.3, 14.9)
November	8.3% (16/193)	(4.4, 12.2)
December	15.8% (29/184)	(10.5, 21.0)

Table 14. Prevalence of *Clostridium difficile* among the human wastewater samples in the 13 units.

Unit	Prevalence	95% Confidence Interval	Unit type
7	17.2% (28/163)	(11.7, 23.9)	farrow-to-finish
3	15.7% (26/166)	(10.5, 22.1)	farrow-to-finish
12	7.6% (13/171)	(4.1, 12.6)	grower-finisher
10	10.1% (20/198)	(6.3, 15.2)	grower-finisher
2	13.0% (21/162)	(8.2, 19.1)	grower-finisher
5	12.8% (20/156)	(8.0, 19.1)	farrow-to-finish
4	10.8% (23/213)	(7.0, 15.8)	grower-finisher
9	13.7% (19/139)	(8.4, 20.5)	grower-finisher
1	10.6% (17/161)	(6.3, 16.4)	farrow-to-finish
11	14.1% (29/206)	(9.6, 19.6)	grower-finisher
8	9.9% (21/212)	(6.2, 14.7)	grower-finisher
6	9.1% (17/187)	(5.4, 14.2)	farrow-to-finish
13	10.8% (17/158)	(6.4, 16.7)	grower-finisher

Table 15. Comparison of the prevalence of *Clostridium difficile* among the human wastewater and swine fecal samples, stratified by the 13 units.

Unit	Swine Prevalence (95% C.I.)	Human Prevalence (95% C.I.)
1	14.6% (11.3, 18.4)	10.6% (6.3, 16.4)
2	6.5% (2.8, 12.3)	13.0% (8.2, 19.1)
3	1.9% (0.8, 4.0)	15.7% (10.5, 22.1)
4	2.5% (0.5, 7.3)	10.8% (7.0, 15.8)
5	10.9% (8.2, 14.2)	12.8% (8.0, 19.1)
6	14.5% (11.2, 18.4)	9.1% (5.4, 14.2)
7	11.6% (8.6, 15.1)	17.2% (11.7, 23.9)
8	4.6% (1.7, 9.8)	9.9% (6.2, 14.7)
9	1.6% (0.2, 5.7)	13.7% (8.4, 20.5)
10	0.9% (0.02, 4.8)	10.1% (6.3, 15.2)
11	4.1% (1.3, 9.3)	14.1% (9.6, 19.6)
12	4.1% (1.8, 8.0)	7.6% (4.1, 12.6)
13	ND[1]	10.8% (6.4, 16.7)

[1]ND = not determined

4.2 Molecular biology results

4.2.1. Swine PCR results

All of the 252 *C. difficile* isolates arising from the swine population were further characterized using PCR for detecting the presence of the *tcdC* gene, presence of the binary toxin gene, presence of the toxin A and toxin B genes, and for toxinotyping. The PCR for the *tcdC* gene is generally thought to be specific for *C. difficile* and, therefore, this test served to eliminate any false positive isolates not ruled out by the API biochemical panel strips. The *tcdC* gene was present in all 252 of our isolates. All isolates belonging to the same toxinotype will have the same *tcdC* gene deletion size; therefore, we sent a representative sample of different toxinotypes for gene sequencing. We found that 250 of our isolates contained the 39-bp deletion and 2 isolates did not contain a deletion in the *tcdC* gene (Table 16). Figure 1 depicts an image of a gel portraying amplified products from the *tcdC* gene PCR. The first five lanes in the gel contain a 100-bp ladder, a wild type strain with no deletion, a toxinotype III strain with an 18-bp deletion, a toxinotype V strain with a 39-bp deletion, and a negative control (not seen), respectively. From the image it can be seen that it is difficult to visually differentiate between the strains with an 18-bp deletion and a 39-bp deletion; this is why gene sequencing of a representative sample of *tcdC* genes from different toxinotypes was necessary. Isolates of the same toxinotype are documented to have some identical characteristics such as size of the *tcdC* gene deletion, presence or absence of the binary toxin gene, or presence or absence of the toxin A and toxin B genes (27) (Table 17).

Table 16. Deletion sizes of the *tcdC* gene among *Clostridium difficile* isolated from the swine fecal samples.

tcdC deletion size	# of isolates
no deletion	2 (0.8%)
18 base pair	0 (0.0%)
39 base pair	250 (99.2%)
Total	252

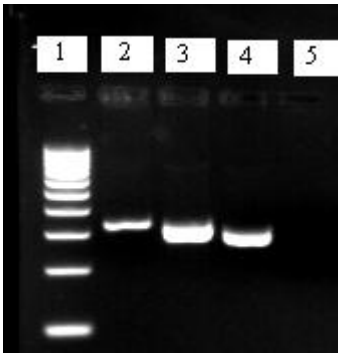

Figure 1. Image of the PCR products for the *tcdC* gene with three controls run on a 2% agarose gel. Lane 1 contains a 100-bp ladder, Lane 2 contains a wild strain with no deletion, Lane 3 contains a toxinotype III with an 18-bp deletion, Lane 4 contains a toxinotype V with a 39-bp deletion, and Lane 5 contains a negative control.

Table 17. Toxinotype results among *Clostridium difficile* isolated from the swine fecal samples.

Toxinotype	Toxin A	Toxin B	Binary toxin	tcdC deletion size	# isolates
V	+	+	+	39 bp	236
V-like	-	+	+	39 bp	7
XI	-	-	+	39 bp	7
Unknown 1	+	+	+	no deletion	2
Total					252

All 252 isolates from the swine population were positive for the binary toxin gene (Figure 2). For the toxin A and toxin B genes, 238 (94.4%) of our isolates were positive for both the toxin A and toxin B genes (A+/B+), 7 (2.8%) were negative for the toxin A gene and positive for the toxin B gene (A-/B+), and 7 (2.8%) were negative for both the toxin A and toxin B genes (A-/B-) (Table 18). *Clostridium difficile* isolates positive for the toxin A gene and negative for the toxin B gene have not been reported (143).

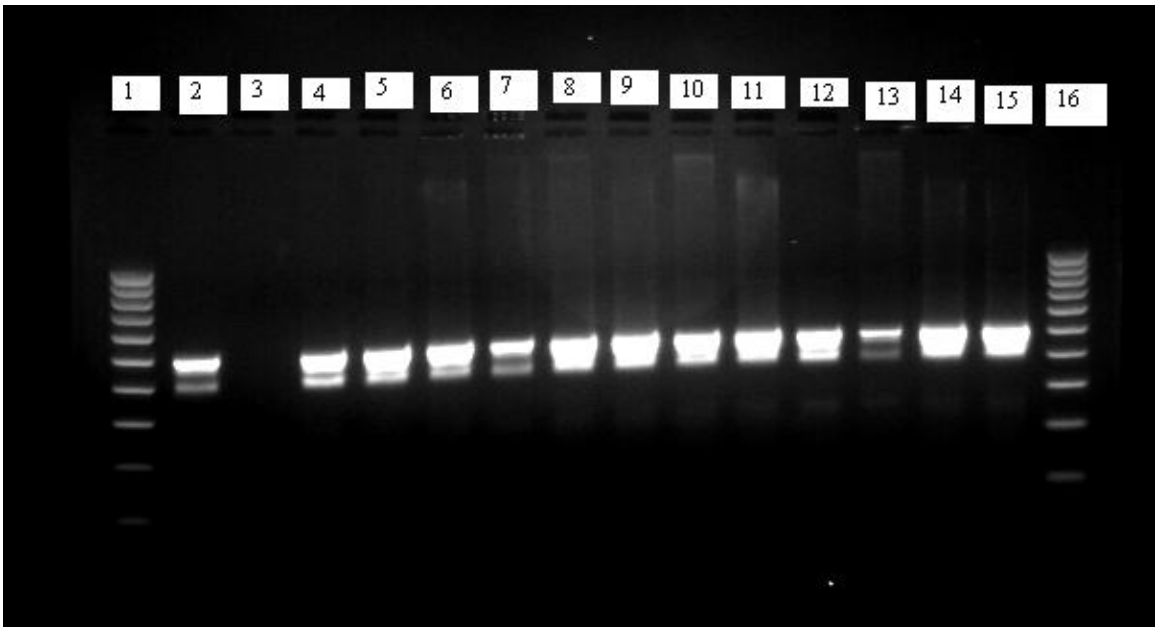

Figure 2. Image of the PCR products for the binary toxin gene run on a 1.5% agarose gel. Lane 1 contains a 100-bp ladder, Lane 2 contains a toxinotype V strain that is known to contain the binary toxin gene, and Lane 3 contains a negative control.

Table 18. Toxin A and toxin B results among *Clostridium difficile* isolated from the swine fecal samples.

Toxin A / B	# isolates
A+ / B+	238
A- / B+	7
A- / B-	7
A+ / B- *	0
Total	252

* This combination has never been reported in the *C. difficile* literature (143).

Figure 3 is a gel image of the amplification products from each of a toxin A and toxin B gene PCR. Lane 1 contains a 100-bp ladder, Lane 2 contains a control strain known to contain toxin A, and Lane 3 contains a control known to contain toxin B. Lanes 4 and 5 as well as 6 and 7 are from isolates that were positive for both the toxin A and toxin B genes (A+/B+). Lanes 8 and 9 are from an isolate that was negative for the toxin A gene and positive for the toxin B gene (A-/B+). Lanes 10 and 11 are from an isolate that was negative for both the toxin A and toxin B genes (A-/B-).

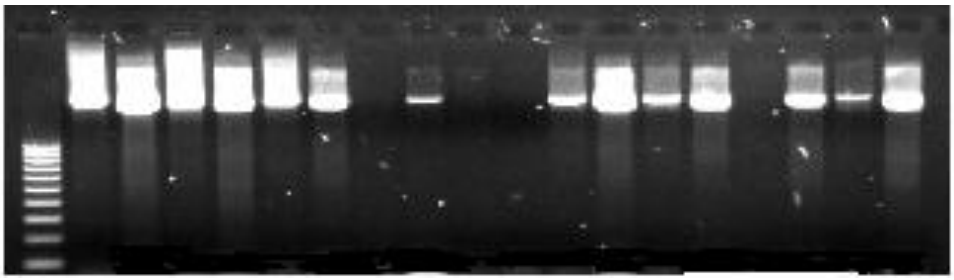

Figure 3. Image of the PCR products for the toxin A and toxin B genes run on a 1.5% agarose gel. Lane 1 contains the 100-bp ladder; Lane 2 and Lane 3 are a toxinotype V strain that is known to be toxin A+/B+; Lanes 8 and 9 are a toxin A-/B+ stain; Lanes 10 and 11 are a toxin A-/B- strain.

The majority of the swine isolates (n=236; 93.7%) were of toxinotype V (Table 17). The other toxinotypes found among the swine isolates included 7 (2.8%) toxinotype V-like, 7 (2.8%) toxinotype XI, and 2 (0.8%) that were of the same unknown toxinotype (Table 17). Toxinotype V isolates are characterized by a 39-bp deletion in the *tcdC* gene, presence of the binary toxin gene, and presence of both toxin A and B genes. Toxinotype V-like isolates have an identical restriction pattern for the toxin B gene as toxinotype V isolates, are positive for the binary toxin gene, and positive for B gene; however, they are negative for the toxin A gene. Figure 4 depicts a toxinotype V alongside a toxinotype V-like isolate for comparison purposes. Lane 1 contains a 100-bp ladder. Lanes 2 through 4 are the *Eco*RI, *Hinc*II, and *Acc*I restriction products for a toxinotype V isolates, respectively. Lanes 5 through 7 are the *Eco*RI, *Hinc*II, and *Acc*I restriction products for a toxinotype V-like isolate, respectively. Toxinotype XI isolates are characterized by a 39-bp deletion in the *tcdC* gene, the presence of the binary toxin gene, and absence of both the toxin A and toxin B genes (Table 17). The two isolates of an unknown toxinotype had no deletion in the *tcdC* gene and were positive for the binary toxin gene, toxin A, and toxin B genes.

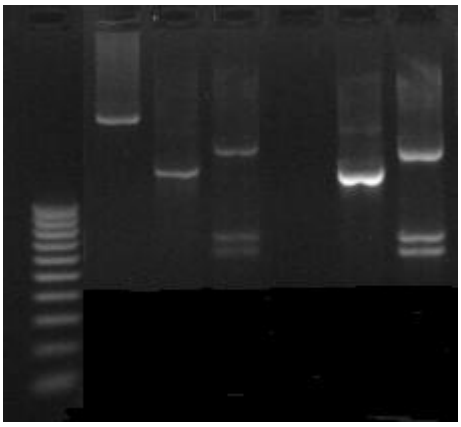

Figure 4. Image of a toxinotype V and toxinotype V-like isolate run on a 1.5% agarose gel. Lane 1 contains a 100-bp ladder. Lanes 2 through 4 contain the restriction products of a toxinotype V isolate of the *Eco*RI, *Hinc*II, and *Acc*I reactions respectively. Lanes 5 through 7 contain the restriction products of a toxinotype V-like isolate of the *Eco*RI, *Hinc*II, and *Acc*I reactions respectively.

There was no significant difference in toxinotypes between units ($p=0.18$) or season ($p=0.07$); however, there was a significant difference ($p<0.05$) in toxinotypes between production groups (Figure 5). Toxinotype V was found in all four of the production group categories whereas toxinotype V-like was only found in the farrowing, grower/finisher, and breeding production groups. Toxinotype XI was found in the farrowing and breeding and the unknown toxinotype was found in the farrowing and grower/finisher production groups. All isolates in the nursery production group were toxinotype V

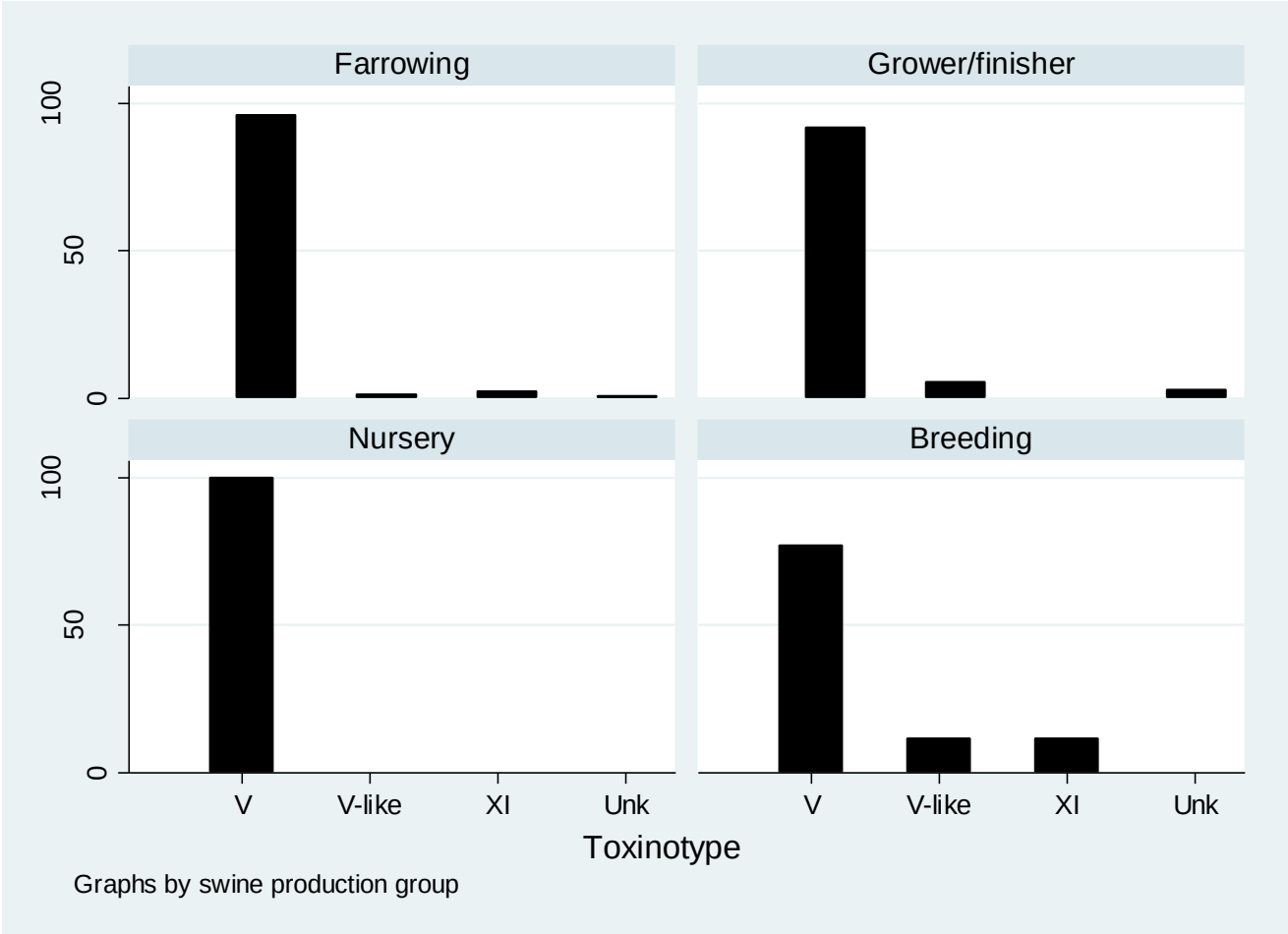

Figure 5. Toxinotypes among swine production groups for the *Clostridium difficile* isolates.

All 252 isolates from the swine population were also characterized using PFGE. We found that 66 (26.2%) of the isolates were PFGE type NAP7 (North American Pulsed-field type 7) (Table 19). The most commonly found PFGE pattern (173 isolates, 68.7%) was a "NAP7-variant" pattern that differed only slightly from the standard NAP7 PFGE pattern by one band. Dendrogram analysis indicated that these patterns were 90.5% similar (Figure 6). There were another 13 isolates that belonged to 4 different unknown PFGE patterns (Table 19). Figure 7 shows a dendrogram of the 6 different PFGE patterns found among the swine isolates. The NAP7 and NAP7-variant patterns are the most similar (90.5%) and Unknown 2 is 86.8% similar to them. Unknown 4, Unknown 1, and Unknown 3 are 82.5%, 70.0%, and 63.0% similar to the NAP7 and variant patterns, respectively. This dendrogram shows that there is a high degree of similarity among the PFGE patterns found in the swine isolates.

Table 19. Pulsed-field gel electrophoresis patterns for positive *Clostridium difficile* isolates from the swine fecal samples.

PFGE	# isolates
NAP7	66
NAP7 variant	173
unknown 1	1
unknown 2	3
unknown 3	8
unknown 4	1
Total	252

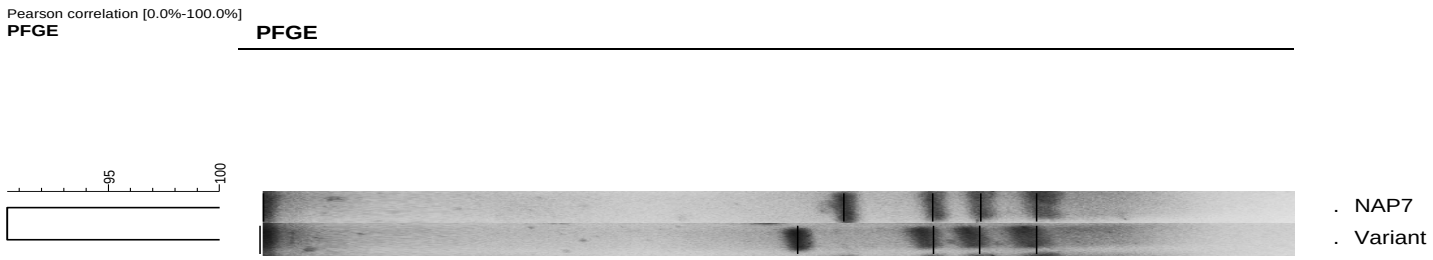

Figure 6. Dendrogram comparing the NAP7 and NAP7-variant PFGE patterns.

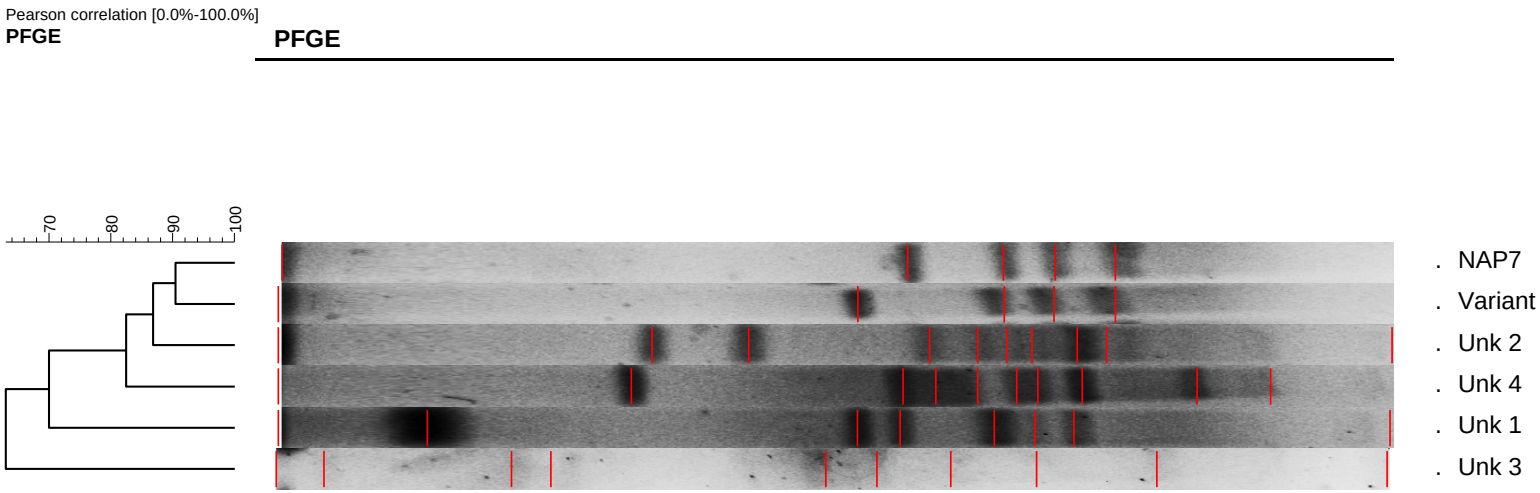

Figure 7. Dendrogram comparing the 6 different PFGE patterns found among the isolates from the swine population.

Figure 8 shows an image of a typical PFGE gel containing restriction enzyme products from swine isolates. Starting in Lane 1, every sixth lane on the gel contains a *Salmonella Braenderup* standard. Lane 2 is a NAP7-variant PFGE pattern, Lane 3 is a NAP7 PFGE pattern, and Lane 21 is Unknown 4 PFGE pattern. Each PFGE pattern can contain several different toxinotypes. A total of 11 unique combinations of PFGE patterns and PCR toxinotypes were found among the 252 swine isolates (Table 20). The majority of the isolates (64.7%) were a combination of the variant PFGE pattern and toxinotype V, followed next by the NAP7 PFGE pattern and toxinotype V combination (25.4%). The remaining 9 combinations were found in a total of less than 5% of the swine fecal isolates. We found that the PFGE pattern did not differ significantly across season ($p=0.25$); however, we did find that PFGE pattern differed significantly ($p<0.05$) across units and production groups when the 4 unknown patterns were collapsed into one category (Figures 9 and 10).

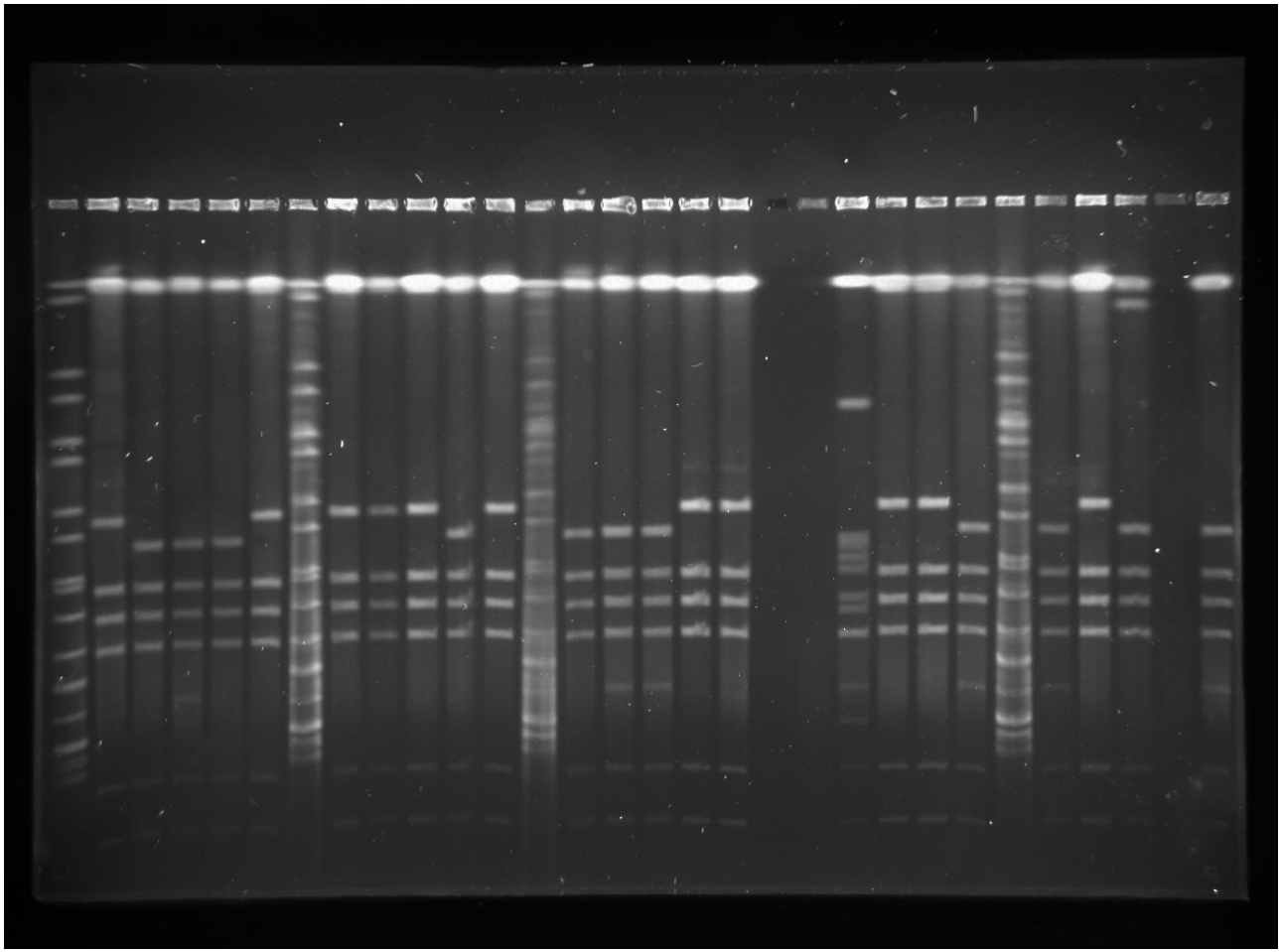

Figure 8. Image of a typical PFGE gel for the isolates from the swine population; Lanes 1, 7, 13, and 25 contain a *Salmonella Braenderup* reference; Lanes 2, 6, 8, 9, 10, 12, 17, 18, 22, 23, and 27 contain the variant pattern; Lanes 3, 4, 5, 11, 14, 15, 16, 24, 26, 28 and 30 contain the NAP7 pattern; Lane 21 contains an unknown pattern.

Table 20. Molecular summary results for positive *Clostridium difficile* isolates from the swine fecal samples.

PFGE	Toxinotype	Toxin A	Toxin B	Binary Toxin	*tcdC* deletion	# isolates
NAP7	V	+	+	+	39 bp	64
NAP7	XI	-	-	+	39 bp	1
NAP7	Unknown 1	+	+	+	No deletion	1
Variant	V	+	+	+	39 bp	163
Variant	V-like	-	+	+	39 bp	7
Variant	XI	-	-	+	39 bp	3
Unknown 1	XI	-	-	+	39 bp	1
Unknown 2	XI	-	-	+	39 bp	2
Unknown 2	V	+	+	+	39 bp	1
Unknown 3	V	+	+	+	39 bp	8
Unknown 4	Unknown 1	+	+	+	No deletion	1
Total						252

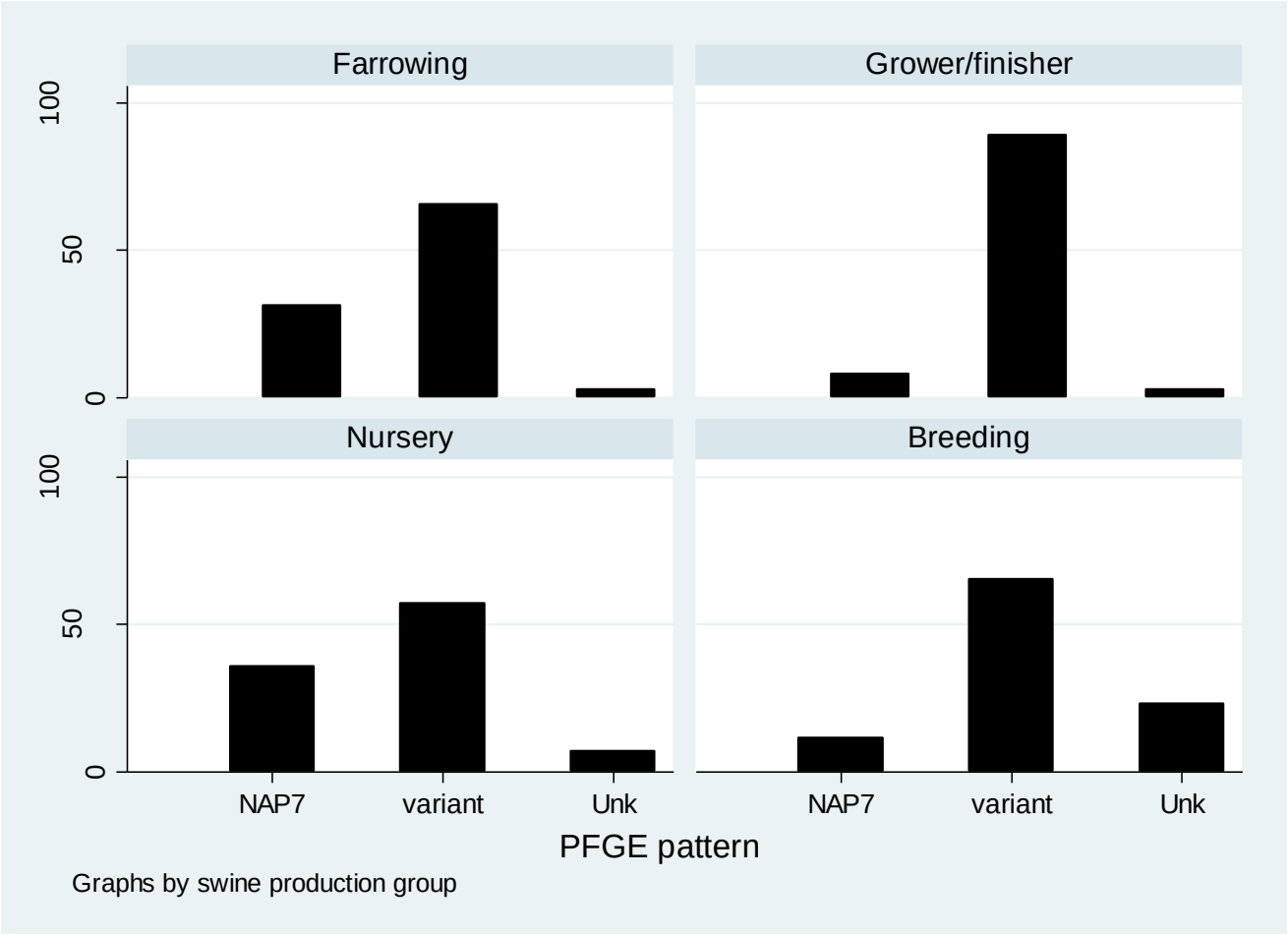

Figure 9. PFGE patterns among swine production groups for *Clostridium difficile* isolates.

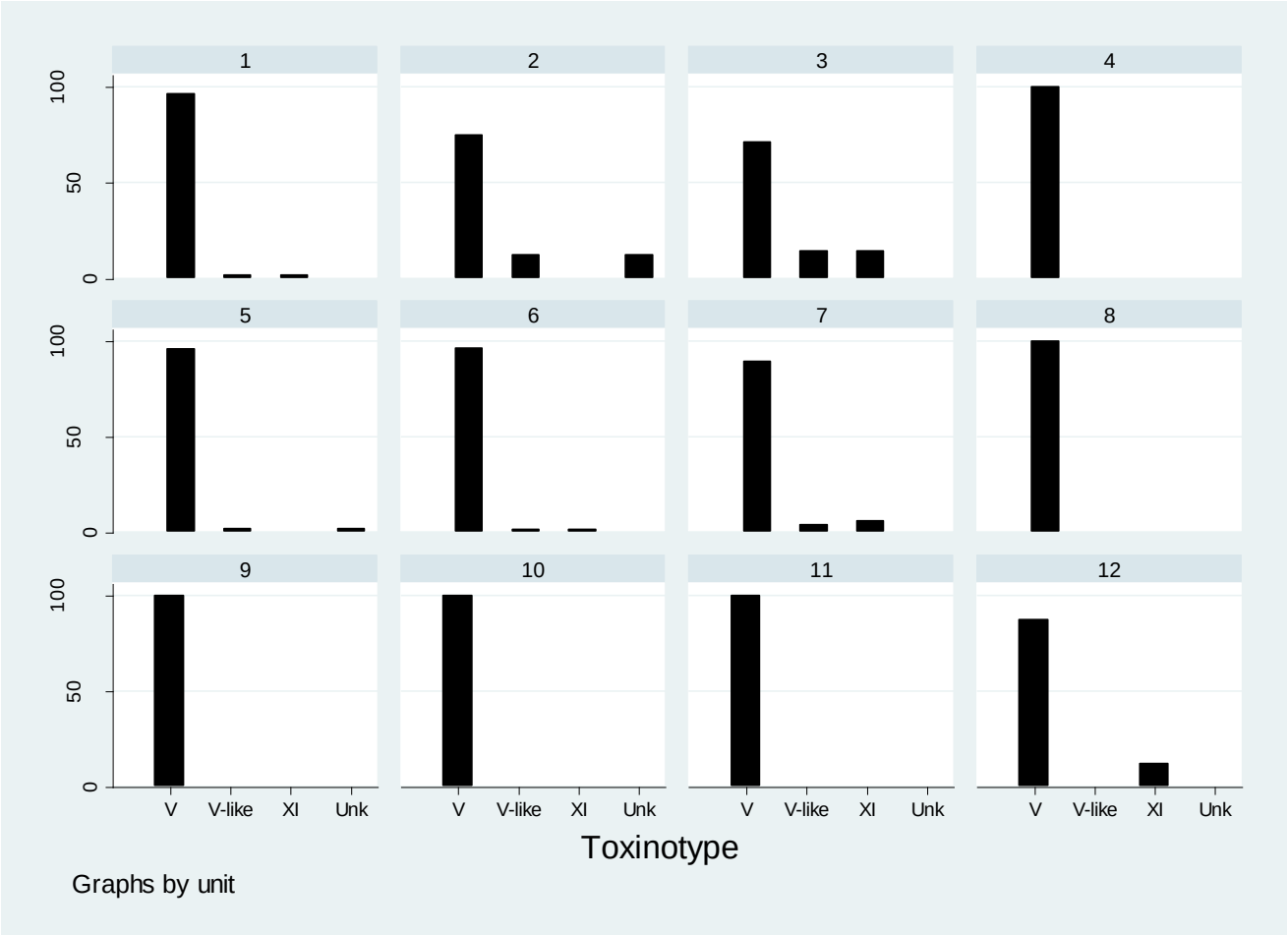

Figure 10. PFGE patterns among the 12 swine units for *Clostridium difficile* isolates.

4.2.2 Human molecular biology results

The 271 *C. difficile* isolates from the human population were further characterized using PCR for the *tcdC* gene, binary toxin gene, toxin A and toxin B genes, and toxinotyping. We found that 244 (90.0%) of our human isolates contained a 39-bp deletion, 26 (9.6%) contained no deletion, and 1 (0.4%) contained an 18-bp deletion in the *tcdC* gene (Table 21). We also found that 244 (90.0%) of our isolates were positive for the binary toxin gene (Table 22). Results from the toxin A and toxin B gene PCR indicated that 259 (95.6%) of the isolates were positive for both the toxin A and toxin B genes (A+/B+), 7 (2.6%) were negative for the toxin A gene and positive for the toxin B gene (A-/B+), and 5 (1.8%) were negative for both the toxin A and toxin B genes (A-/B-) (Table 23).

Table 21. Deletion sizes of the *tcdC* gene for *Clostridium difficile* isolates from culture-positive human wastewater samples.

tcdC deletion size	# of isolates
no deletion	26
18 base pair	1
39 base pair	244
Total	271

Table 22. Binary toxin results for *Clostridium difficile* isolates arising from the human wastewater samples.

Binary Toxin	# of isolates
positive	244
negative	27
Total	271

Table 23. Toxin A and toxin B results for *Clostridium difficile* isolates arising from the human wastewater samples.

Toxin A / B	# isolates
A+ / B+	259
A- / B+	7
A- / B-	5
A+ / B- *	0
Total	271

* This combination has not been previously reported in the *C. difficile* literature (143).

PCR toxinotyping indicated that 229 (84.5%) of our isolates were toxinotype V, 7 (2.6%) were toxinotype V-like, 5 (1.8%) were toxinotype XI, 1 was toxinotype III, 6 (2.2%) were of a single unknown toxinotype (Unknown 2), 3 (1.1%) were of another unknown toxinotype (Unknown 3), and 20 (7.4%) were a third unknown toxinotype (Unknown 4) (Table 24). Toxinotype III isolates are characterized by an 18-bp deletion in the *tcdC* gene, presence of the binary toxin gene, and presence of the toxin A and toxin B genes. Unknown toxinotype 2 isolates had no *tcdC* gene deletion, were negative for the binary toxin gene, and were positive for the toxin A and toxin B genes. Unknown toxinotype 3 isolates had a 39-bp deletion in the *tcdC* gene, were positive for the binary toxin, toxin A, and toxin B genes, but had a different restriction pattern than the toxinotype V isolates. Unknown toxinotype 4 isolates had no *tcdC* gene deletion, were negative for the binary toxin, were positive for the toxin A and toxin B genes, and had a different restriction pattern than unknown 2 (Table 24). There was no significant difference in toxinotypes across season ($p=0.12$), unit ($p=0.21$), or occupational group cohort ($p=0.48$).

Table 24. Toxinotype results for *Clostridium difficile* isolates arising from the human wastewater samples.

Toxinotype	Toxin A	Toxin B	Binary toxin	*tcdC* deletion size	# isolates
V	+	+	+	39bp	229
V-like	-	+	+	39bp	7
XI	-	-	-	39bp	5
III	+	+	+	18bp	1
Unknown 2	+	+	-	no deletion	6
Unknown 3	+	+	+	39 bp	3
Unknown 4	+	+	-	no deletion	20
Total					271

Among the isolates from the human population, seventeen different PFGE patterns were identified. The two most common PFGE patterns were NAP7 (23.6%) and a variant pattern (66.8%) that was 90.5% similar to the NAP7 pattern (Table 25). The remaining 26 isolates belonged to 15 different unknown patterns (Table 25). There was only one isolate for each of nine of the 15 patterns. A dendrogram of the 17 different patterns showed that many of the unknown PFGE patterns in the human isolates were very similar (Figure 11). Unknown 12 and Unknown 17 are the most similar (96.3%) and Unknown 14 and Unknown 9 are 94.4% and 93.0% similar, respectively, to unknown 12 and unknown 17. There were two major clusters among the human isolates and the isolates in each cluster were more than 80% similar. Unknown 3 was an outlier from the two clusters and showed the least similarity with the other isolates (75.5%). There was no significant difference in PFGE patterns among seasons ($p=0.06$), units ($p=0.39$), or occupational group cohorts ($p=0.77$).

Table 25. Pulsed-field gel electrophoresis results for *Clostridium difficile* isolates arising from the human wastewater samples. See Figure 12 for images of a selection of unknown patterns.

PFGE	# isolates
NAP7	64
Variant	181
Unknown 3	6
Unknown 4	2
Unknown 5	2
Unknown 6	1
Unknown 7	2
Unknown 8	2
Unknown 9	3
Unknown 10	1
Unknown 11	1
Unknown 12	1
Unknown 13	1
Unknown 14	1
Unknown 15	1
Unknown 16	1
Unknown 17	1
Total	271

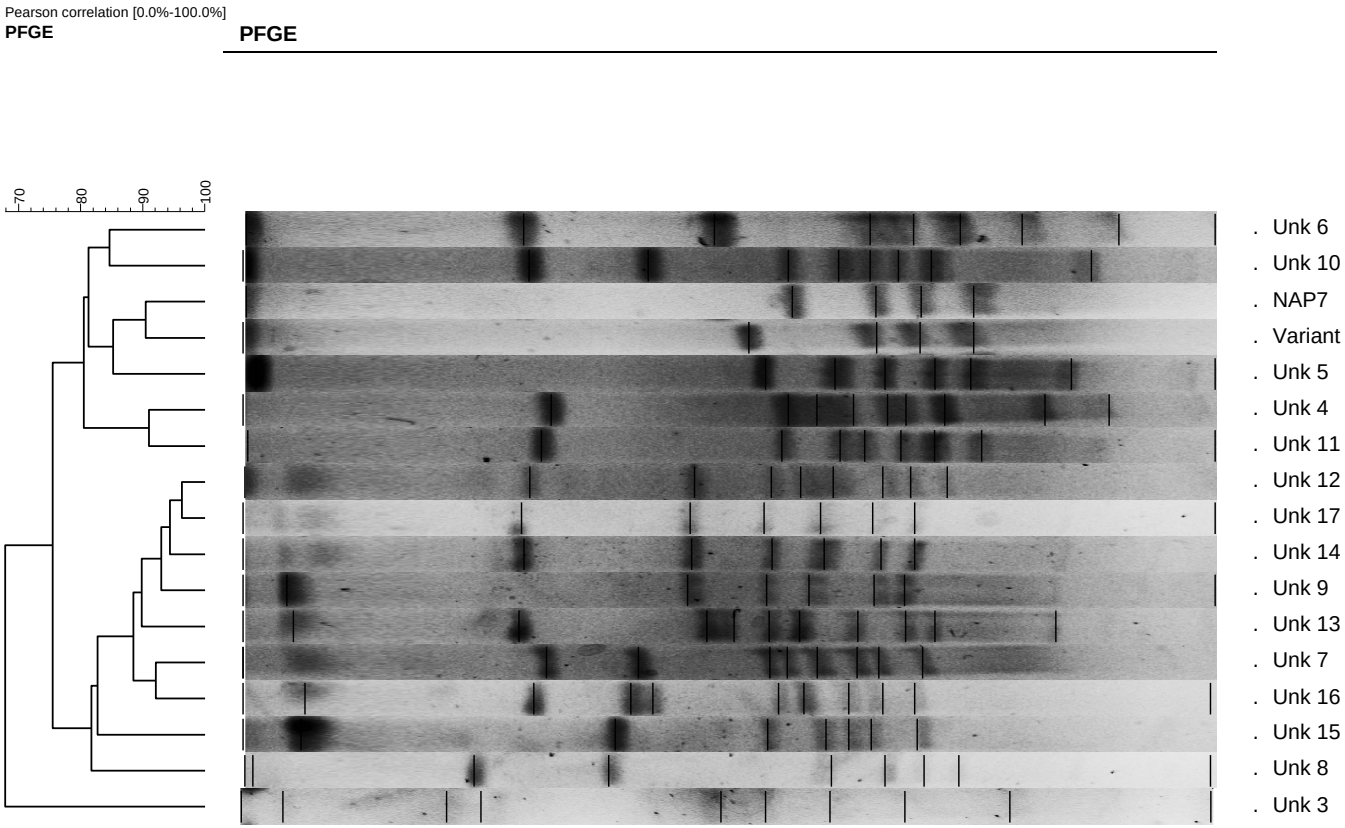

Figure 11. Dendrogram comparing the 17 different PFGE patterns found in the *Clostridiium difficile* isolates from the human population.

Figure 12 shows a typical image from a PFGE gel arising from the human isolates. Starting with the first lane, every sixth lane contains a *Salmonella Braenderup* reference. Lane 2 is a variant pattern, Lane 20 is an NAP7 isolate, and Lanes 6, 8, 9, 16, 17, 18, and 27 show some of the unknown PFGE patterns. The human population isolates represented a greater diversity of toxinotypes and PFGE patterns than swine; among the human isolates there were 29 unique combinations of PFGE patterns and toxinotypes (Table 26). The majority of the isolates (61.6%) were of the variant (NAP7) PFGE pattern and toxinotype V followed by the NAP7 PFGE pattern and toxinotype V combination (22.1%). The remaining 27 combinations were found in less than 2% of the isolates arising from the human population.

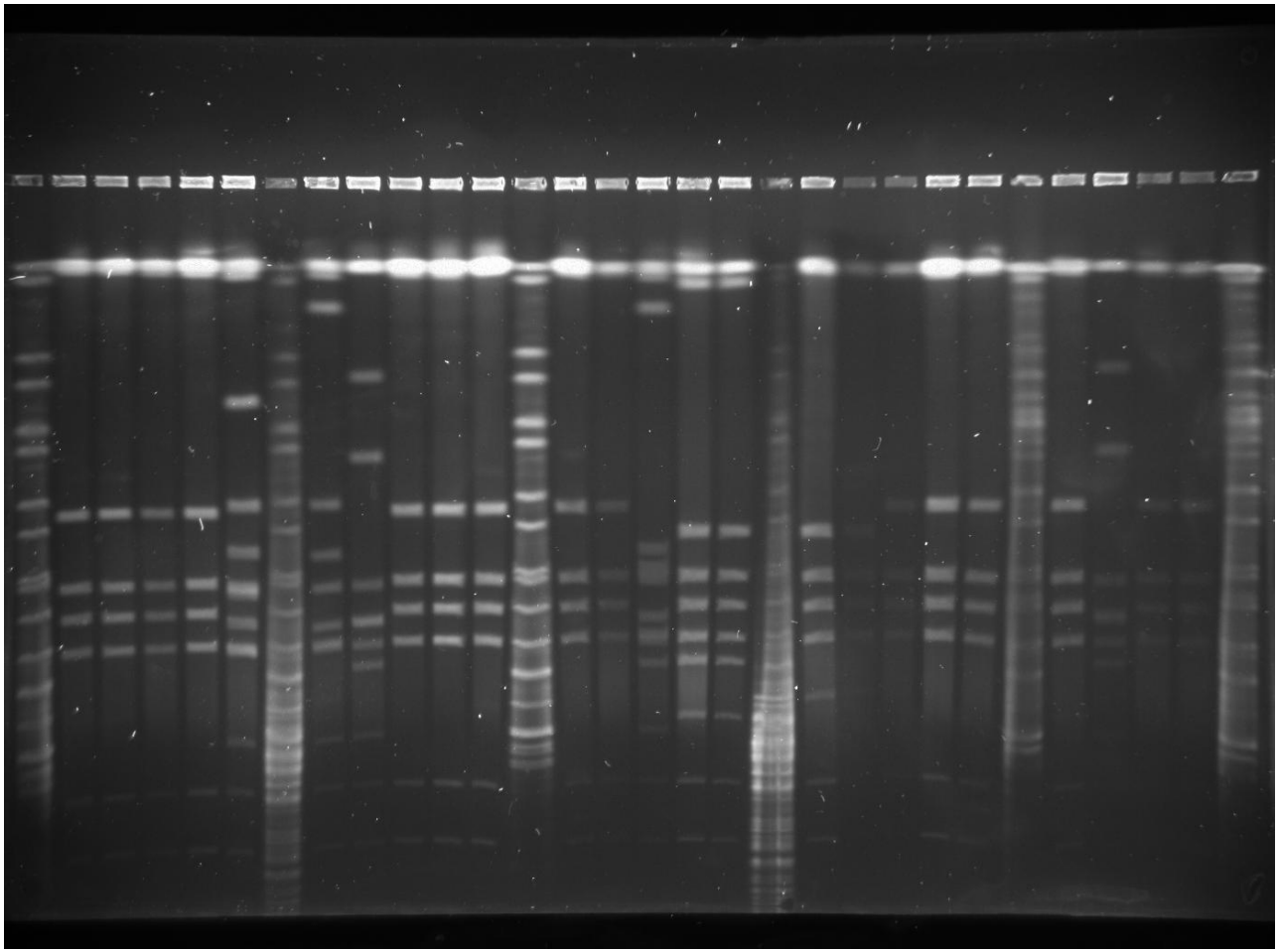

Figure 12. PFGE gel image of restriction enzyme fragments of *Clostridium difficile* isolates arising from the human population. Lanes 1, 7, 13, 19, 25, and 30 contain the *Salmonella Braenderup* reference. Lanes 2, 3, 4, 5, 10, 11, 12, 14, 15, 22, 23, 24, 26, 28, and 29 contain the variant pattern; Lanes 20 and 21 contain the NAP7 pattern; Lane 6 contains Unknown 14; Lane 8 contains Unknown 9; Lanes 9 and 27 contain Unknown 16. Lanes 17 and 18 contain Unknown 5.

Table 26. Molecular summary results for *Clostridium difficile* isolates arising from the human wastewater samples

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PFGE	Toxinotype	Toxin A	Toxin B	Binary Toxin	*tcdC* deletion	# isolates
NAP7	V	+	+	+	39-bp	60
NAP7	V-like	-	+	+	39-bp	3
NAP7	Unknown 3	+	+	+	39-bp	1
Variant	V	+	+	+	39-bp	167
Variant	V-like	-	+	+	39-bp	4
Variant	XI	-	-	+	39-bp	5
Variant	III	+	+	+	18-bp	1
Variant	Unknown 2	+	+	-	no deletion	2
Variant	Unknown 3	+	+	+	39-bp	1
Variant	Unknown 4	+	+	-	no deletion	1
Unknown 3	Unknown 2	+	+	-	no deletion	1
Unknown 3	Unknown 4	+	+	-	no deletion	5
Unknown 4	Unknown 2	+	+	-	no deletion	1
Unknown 4	Unknown 4	+	+	-	no deletion	1
Unknown 5	V	+	+	+	39-bp	2
Unknown 6	Unknown 4	+	+	-	no deletion	1
Unknown 7	Unknown 2	+	+	-	no deletion	1
Unknown 7	Unknown 4	+	+	-	no deletion	1
Unknown 8	Unknown 2	+	+	-	no deletion	1
Unknown 8	Unknown 4	+	+	-	no deletion	1
Unknown 9	Unknown 4	+	+	-	no deletion	3
Unknown 10	Unknown 4	+	+	-	no deletion	1
Unknown 11	Unknown 4	+	+	-	no deletion	1
Unknown 12	Unknown 4	+	+	-	no deletion	1
Unknown 13	Unknown 3	+	+	+	39-bp	1
Unknown 14	Unknown 4	+	+	-	no deletion	1
Unknown 15	Unknown 4	+	+	-	no deletion	1
Unknown 16	Unknown 4	+	+	-	no deletion	1
Unknown 17	Unknown 4	+	+	-	no deletion	1
Total						271

4.2.3 Comparison of molecular results between host species

The majority of the isolates in the swine population and the human population shared the same characteristics. In both populations, the most prevalent toxinotype was toxinotype V (Figure 13) and the most prevalent PFGE type was the variant pattern (Figure 14). Isolates that were the variant PFGE pattern and toxinotype V all had a 39-bp deletion in the *tcdC* gene, were positive for the binary toxin gene, and were positive for the toxin A and toxin B genes. Between host species there was a significant difference in the size of the *tcdC* gene deletion ($p<0.001$), presence or absence of the binary toxin gene ($p<0.001$), and toxinotype ($p<0.001$). No significant difference between host species was found for the PFGE patterns ($p=0.15$), presence or absence of the toxin A gene ($p=0.69$), and presence or absence of the toxin B gene ($p=0.57$). Overall, there was a larger diversity of PFGE patterns and toxinotypes in the human population; however, the majority of the combinations that were unique to the human population were only represented by a single isolate (Table 27). Figure 15 is a dendrogram showing the 19 different PFGE patterns found in both the human and swine populations. Unknown PFGE patterns 1 and 2 were unique to the swine population, whereas Unknown patterns 5 through 17 were unique to the human population. Unknown PFGE patterns 3 and 4 were found in both the human and swine population. Interestingly, the Unknowns that were found in both populations were most similar to an Unknown found in only one of the host species.

Unknown 4 was 91% similar to Unknown 11, which was only found in the human population while Unknown 3 was 69.9% similar to Unknown 1, which was found only in the swine population. When comparing Unknown isolates 1, 2, 3 and 4 in the dendrogram with only swine patterns (Figure 7) to the dendrogram with all 19 patterns (Figure 15), it is evident these isolates were more closely related to isolates found only in human population than to isolates found only in the swine population. As in the dendrogram of the human isolates, there were 2 main clusters and within these clusters the isolates were more than 80% similar. Unknown PFGE patterns 1 and 3 were the outliers in a separate cluster. These patterns were 69.9% similar to each other and were 75.4% similar to the other two clusters. This dendrogram is evidence that there was a high degree of similarity between our isolates and many of the 19 patterns were clonal, using 80% similarity as the cutoff for clonality.

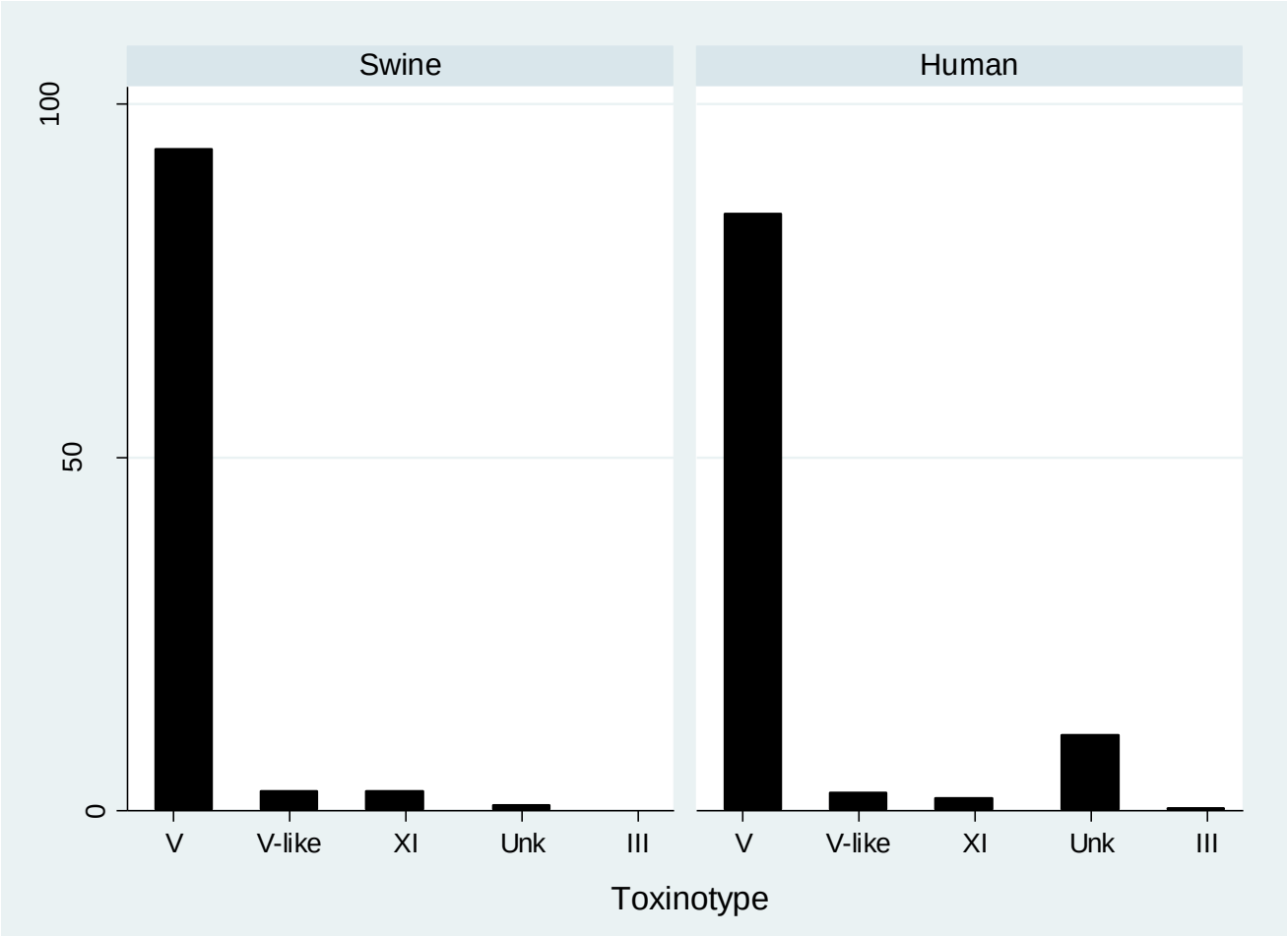

Figure 13. Comparison of *Clostridium difficile* PCR toxinotyping results between the host species.

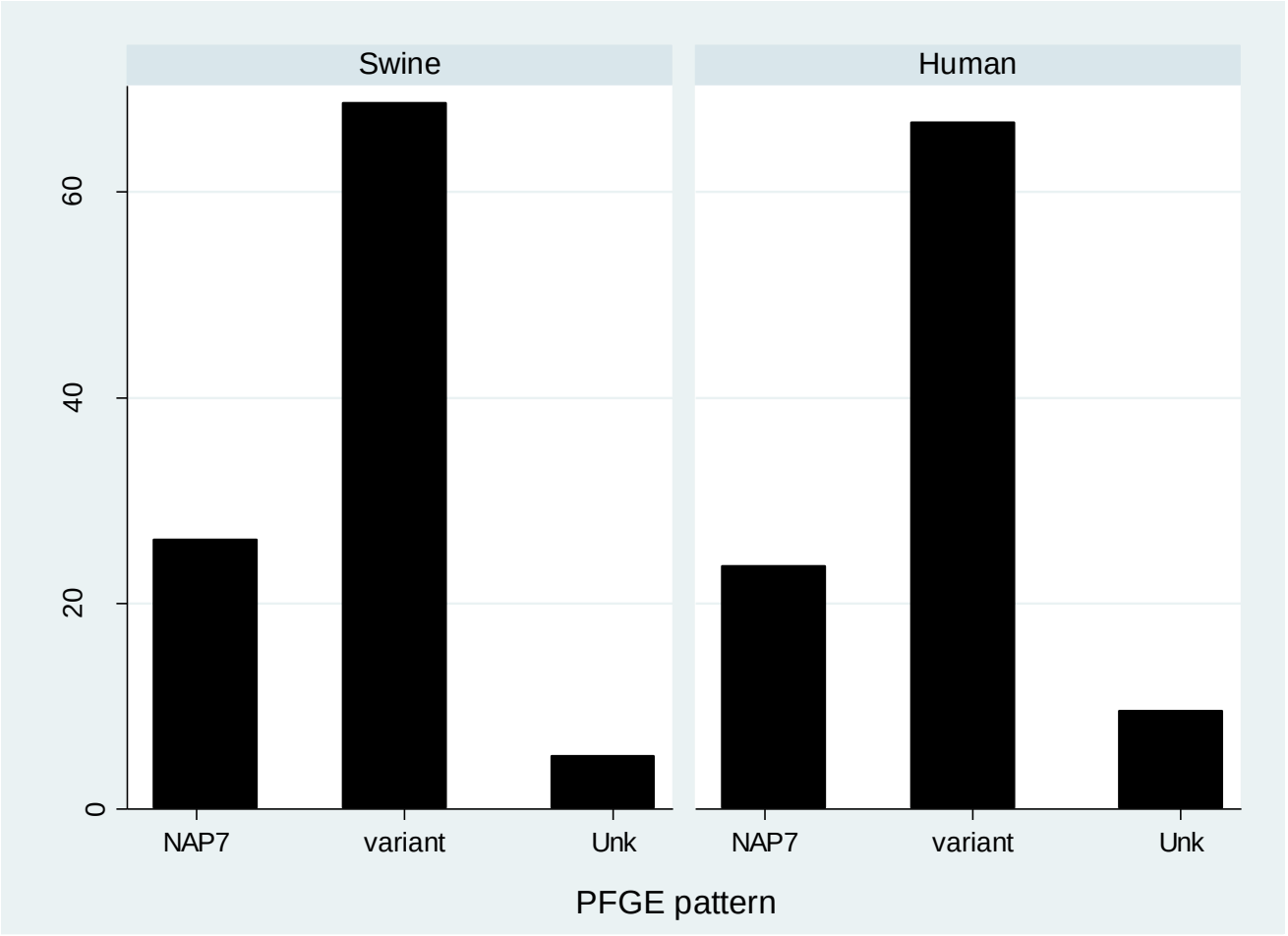

Figure 14. Comparison of *Clostridium difficile* pulsed-field gel electrophoresis results between the host species.

Table 27. Comparison of *Clostridium difficile* PFGE/toxinotype combinations between the host species.

Human			Swine		
PFGE	Toxinotype	# isolates	PFGE	Toxinotype	# isolates
NAP7	V	60	NAP7	V	64
NAP7	V-like	3	NAP7	XI	1
NAP7	Unknown 3	1	NAP7	Unknown 1	1
Variant	V	167	Variant	V	163
Variant	V-like	4	Variant	V-like	7
Variant	XI	5	Variant	XI	3
Variant	III	1	Unknown 1	XI	1
Variant	Unknown 2	2	Unknown 2	XI	2
Variant	Unknown 3	1	Unknown 2	V	1
Variant	Unknown 4	1	Unknown 3	V	8
Unknown 3	Unknown 2	1	Unknown 4	Unknown 1	1
Unknown 3	Unknown 4	5			
Unknown 4	Unknown 2	1	Total		252
Unknown 4	Unknown 4	1			
Unknown 5	V	2			
Unknown 6	Unknown 4	1			
Unknown 7	Unknown 2	1			
Unknown 7	Unknown 4	1			
Unknown 8	Unknown 2	1			
Unknown 8	Unknown 4	1			
Unknown 9	Unknown 4	3			
Unknown 10	Unknown 4	1			
Unknown 11	Unknown 4	1			
Unknown 12	Unknown 4	1			
Unknown 13	Unknown 3	1			
Unknown 14	Unknown 4	1			
Unknown 15	Unknown 4	1			
Unknown 16	Unknown 4	1			
Unknown 17	Unknown 4	1			
Total		271			

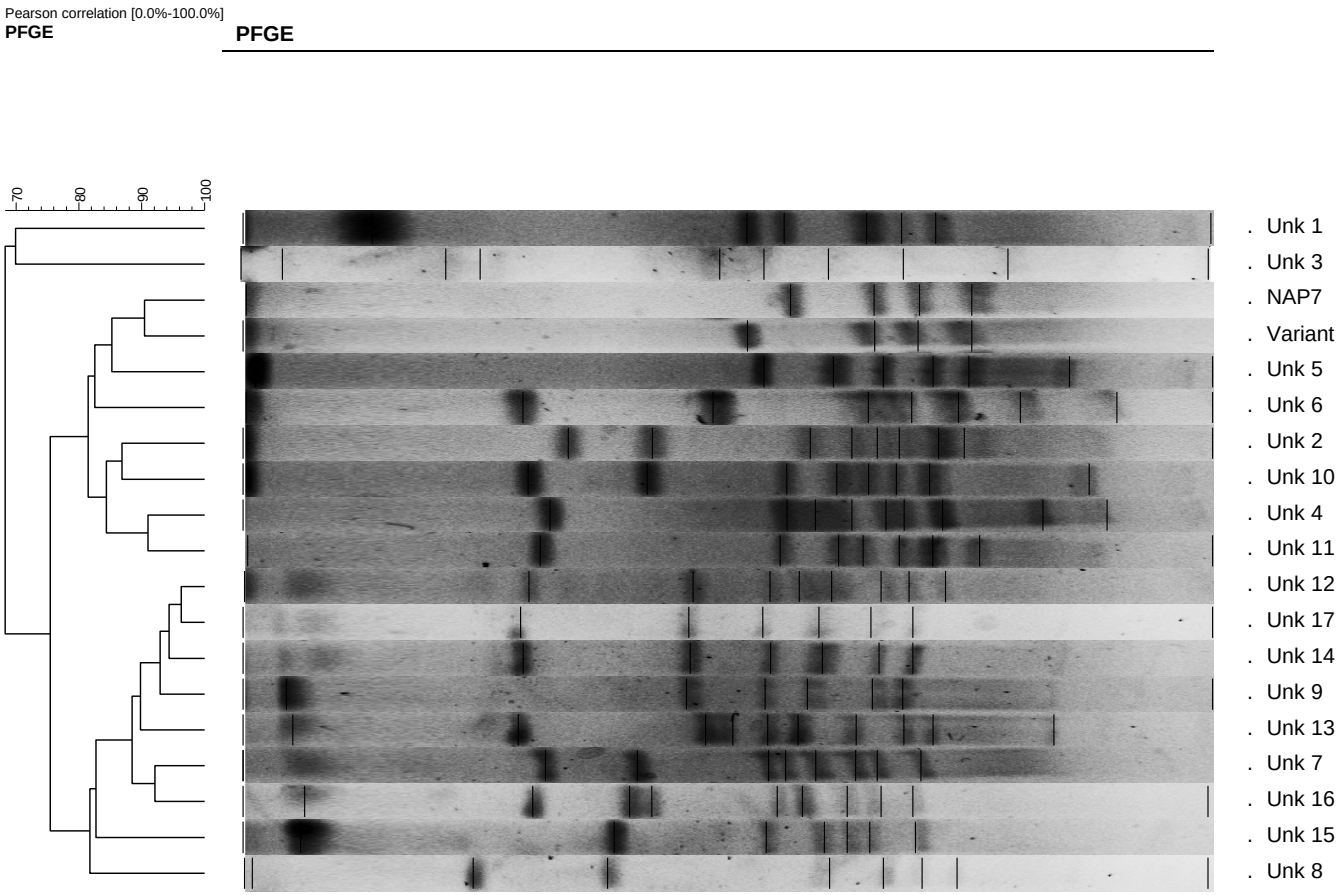

Figure 15. Dendrogram comparing all 19 PFGE patterns found in both the swine and human populations.

4.3 Multivariable logistic regression models

4.3.1 Multivariable and multilevel mixed-effects logistic regression models for the swine population

Using a multivariable logistic regression model we tested if there was an important association between the fixed effects of production group, month, year, and season (and the interaction of these terms) and *C. difficile* prevalence in the swine population. The random effect in the model was the unit itself, in order to account for the inherent dependency of responses by location. Production group was significant ($p<0.001$), while season ($p=0.94$) and month ($p=0.37$) were not significantly associated with prevalence of *C. difficile*. Sampling year contributed significantly to the model as a fixed effect ($p<0.001$). The final model including unit as a random effect retained production group and year as the significant fixed effects (Table 28). The interaction of production group and year was not found to be significant ($p=0.15$). Since sampling year was significant to the first multivariable model, a multilevel, mixed-effects logistic regression model was built that took into account both unit and year as random effects to account for the dependency of responses by location and over time. Production group remained significant ($p<0.001$) when unit and year were included as random effects (Table 29). As in the previous model, season ($p=0.83$) and month ($p=0.31$) were unimportant.

A large component of the variance for *C. difficile* prevalence initially attributed to the units themselves was explained by the difference in production group types across

the units (i.e., farrow-to-finish versus grower-to-finisher). Once these fixed effects were included in the models the variance component (i.e., random effect) associated with unit was diminished greatly. In the intercept-only model 54.4% of the variance was attributed to the unit, whereas in the final model that included the production groups only 32.3% of the variance was attributed to the unit. However, the addition of production group to the model did not help explain any variance in *C. difficile* prevalence between years. In the intercept only model 34.3% of the variance was attributed to year, whereas in the final model with production groups 37.1% of the variance was attributed to year. When comparing the models with year as a random effect versus as a fixed effect there was little meaningful difference between the estimated coefficients (and adjusted odds ratios) for the production group fixed effects.

Table 28. Coefficients and odds ratios from multivariable logistic regression model of the swine population with unit as a random effect.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	Odds Ratio 95% Confidence Interval
Intercept			-0.093	-	-
Swine production group	226.01; p<0.001 (3 d.f)	farrowing(referent category)	-	-	-
		grower/finisher	-2.75	0.06	0.04--0.11
		nursery	-1.98	0.14	0.08--0.25
		breeding	-2.04	0.13	0.08--0.21
Year	54.74; p<0.001 (2 d.f.)	2004 (referent category)	-	-	-
		2005	-1.00	0.37	0.24--0.56
		2006	0.38	1.46	1.06--2.01

LR test of random vs. fixed effect logistic regression: $\chi^2_{1\ d.f.} = 39.10$; P<0.00001
Intercept only model – 54.4% of variance attributed to random effect of the unit.
Final model – 32.3% of variance attributed to random effect the unit.

Table 29. Coefficients and odds ratios from the multilevel mixed-effects logistic regression model of the swine population with unit and year treated as random effects.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	Odds Ratio 95% Confidence Interval
Intercept			-1.13	-	-
Swine production group	225.28; p<0.001 (3 d.f)	farrowing(referent category)	-	-	-
		grower/finisher	-2.74	0.06	0.04--0.11
		nursery	-1.96	0.14	0.08--0.25
		breeding	-2.06	0.13	0.08--0.20

LR test of random vs. fixed effect logistic regression: $\chi^2_{2\ d.f} = 67.72$; P<0.00001
Intercept only model – 54.4% of variance attributed to random effect of the unit and 34.3% attributed to random effect of the year.
Final model – 32.3% of variance attributed to random effect of the unit and 37.1% attributed to random effect of the year.

4.3.2 Multivariable and multilevel mixed-effects logistic regression models for the human population

A multivariable logistic regression model was developed to test if there was an association between the fixed factors of occupational group cohort, month, season, year, and the 2-way interactions of these terms with the prevalence of *C. difficile* in the human population. The random effect in the model was the unit to account for the dependency of responses by location. The occupational group cohort in the human population was not significantly ($p=0.81$) associated with prevalence. Season ($p=0.002$), month ($p<0.001$), and year ($p<0.001$) were all significant. The variable season contained the same data as month, except it was collapsed into the four categories winter, spring, summer, and fall. Winter included December, January, and February; spring included March, April, and May; summer included June, July, and August; and fall included September, October, and November. Since season was significantly associated with prevalence in bivariable analyses, this variable was included in the final model, rather than month, because there would be a larger number of observations in each category and therefore would be fewer degrees of freedom employed in the fixed portion of the model. The final multivariable logistic regression model with unit as the random effect included season and year as fixed factors (Table 30).

As with the swine population, sampling year was significantly associated with prevalence ($p<0.001$) and, therefore, a multilevel mixed-effects logistic regression model was run that took into account both unit and year as random effects in the model in order to account for the dependency of responses by location and repeated over time. The occupational group cohort remained not significant ($p=0.93$); however, season ($p=0.002$) and month ($p<0.001$) were significant when unit and year were included as random effects in univariate analysis. The final multilevel mixed-effects logistic regression model for the human population with unit and year as random effects included season as a fixed effect rather than month (Table 31). The variance in *C. difficile* prevalence attributed to the unit (random effect) was small (< 1.0%) and was not greatly affected by adding the fixed effect of season to the model. The variance in *C. difficile* attributed to the year (21.8%) was greater than that attributed to the unit, but was also not affected by adding season to the model (23.6%).

Table 30. Coefficients and odds ratios from multivariable logistic regression model of the human population with unit as a random effect.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	Odds Ratio 95% Confidence Interval
Intercept			-2.31	-	-
	15.96; p=0.001				
Season	(3 d.f)	winter (referent category)	-	-	-
		spring	0.51	1.67	1.17--2.38
		summer	-0.03	0.97	0.66--1.42
		fall	-0.13	0.88	0.60--1.29
	63.57; p<0.001				
Year	(2 d.f.)	2004 (referent category)	-	-	-
		2005	0.74	2.09	1.56--2.79
		2006	-0.60	0.55	0.37--0.82

LR test of random vs. fixed effect logistic regression: $\chi^2_{1\ d.f} = 0.15$; P<0.35
Intercept only model – less than 1.0% of variance attributed to random effect of the unit.
Final model – less than 1% of variance attributed to random effect of the unit.

Table 31. Coefficients and odds ratios from the multilevel mixed-effects logistic regression model of the human population with unit and year as the random effects.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	Odds Ratio 95% Confidence Interval
Intercept			-2.22	-	-
Season	15.47; p=0.002 (3 d.f)	winter (referent category)	-	-	-
		spring	0.51	1.67	1.17--2.37
		summer	-0.02	0.98	0.67--1.43
		fall	-0.12	0.88	0.60--1.30

LR test of random vs. fixed effect logistic regression: $\chi^2_{2\ d.f} = 19.05$; P=0.0001
Intercept only model – less than 1.0% of variance attributed to random effect of the unit and 21.8% attributed to random effect of the year.
Final model – less than 1% of variance attributed to random effect of the unit and 23.6% attributed to random effect of the year.

4.3.3 Multilevel mixed effects model for phenotypes across host species

A multilevel mixed-effects model was used to test the association between the fixed factors of either host species or swine production group/human occupational group cohort (collinear/nested with host species), season, and month and the interaction of these factors and *C. difficile* prevalence in this closed population. Unit and year were included as random factors in the model to account for the dependency of responses by location and over time. Host species ($p=0.05$) or swine production group/human occupational group cohort ($p<0.001$) were significant predictors of *C. difficile* prevalence. Season ($p=0.16$) and month ($p=0.08$) individually were not significantly associated with *C. difficile* prevalence. However, when season was added as a fixed factor with either host species or production group/group cohort we found that both factors were significantly associated with prevalence ($p<0.001$). The interaction terms of host species and season were also significant ($p<0.05$). On the other hand, the interaction terms of swine production group/ human occupational group cohort and season were not significant ($p=0.06$). Host species and swine production group/ human occupational group cohort were not included in the same model, because of collinearity and, therefore, two different final models were presented.

From the models in the previous sections it is evident that season was significant in the human population, but not in the swine population. Therefore, season was forced into the final models while month was omitted. The first model included the fixed effect of host species, season, and the interaction of host species and season (Table 32). The second model included the fixed effect of swine production group / human occupational group cohort and season with swine farrowing as the referent group for the cohort type and winter as the referent group for season (Table 33). As in the multivariable logistic regression model for the swine population, the addition of the swine production group/human occupational group cohort reduced the variance attributed to the unit. In the intercept only model, the variance attributed to the unit was 4.7% and in the final model with swine production group/human occupational group cohort as a fixed factor the variance associated with unit was 1.9%.

Table 32. Coefficients and odds ratios from the multilevel mixed-effects logistic regression model for both host species; with host species, season, and the interaction of host species and season as the fixed factors and unit and time treated as the random effects.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	Odds Ratio 95% Confidence Interval
Intercept			-2.57	-	-
	3.98; p=0.05				
host species	(1 d.f)	swine (referent category)	-	-	-
		human	0.39	1.48	1.01--2.17
	0.45; p=0.93				
season	(3 d.f.)	winter (referent category)	-	-	-
		spring	-0.04	0.96	0.67--1.38
		summer	-0.03	0.97	0.66--1.41
		fall	0.07	1.07	0.74--1.56
	9.14; p=0.03				
host species and season interaction	(3 d.f.)	swine winter	-	-	-
		human winter	-	-	-
		swine spring	-	-	-
		human spring	0.56	1.75	1.06--2.91
		swine summer	-	-	-
		human summer	0.06	1.06	0.62--1.81
		swine fall	-	-	-
		human fall	-0.15	0.86	0.50--1.46

LR test of random vs. fixed effect logistic regression: $\chi^2_{2\ d.f} = 37.62$; P<0.00001
Intercept only model – 4.7% of variance attributed to random effect of the unit and 7.4% attributed to random effect of the year.
Final model – 7.9% of variance attributed to random effect of the unit and 9.3% attributed to random effect of the year.

Table 33. Coefficients and odds ratios from the multilevel mixed-effects logistic regression model for both host species; with swine production group/human occupational group cohort and season as the fixed factors and unit and time treated as the random effects.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	Odds Ratio 95% Confidence Interval
Intercept			-1.25	-	-
	270.34; p<0.001				
group cohort	(5 d.f)	farrowing (referent category)	-	-	-
		grower/finisher	-2.46	0.09	0.06--0.13
		nursery	-1.85	0.16	0.09--0.28
		breeding	-1.98	0.14	0.09--0.21
		swine non-worker	-0.87	0.42	0.32--0.55
		swine worker	-0.84	0.43	0.33--0.57
	5.49; p=0.14				
season	(3 d.f.)	winter (referent category)	-	-	-
		spring	0.25	1.29	1.00--1.67
		summer	0.04	1.04	0.79--1.37
		fall	-0.01	0.99	0.75--1.30

LR test of random vs. fixed effect logistic regression: $\chi^2_{2\ d.f} = 13.50$; P=0.001
Intercept only model – 4.7% of variance attributed to random effect of the unit and 7.4% attributed to random effect of the year.
Final model – 1.9% of variance attributed to random effect of the unit and 7.3% attributed to random effect of the year.

4.4 Antimicrobial resistance data

4.4.1 Swine binary antimicrobial resistance data

All 252 of the swine isolates were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, and vancomycin (100% susceptibility, 0% resistance). The majority of the swine isolates were susceptible to ampicillin (84.9%), chloramphenical (99.2%), clindamycin (56.0%), metronidazole (91.7%), and tetracycline (98.0%) (Table 34). The majority had decreased susceptiblity to cefoxitin (95.6%), ciprofloxacin (86.5%), and imipenem (58.7%) (Table 34). In unconditional associations, there was a significant difference in susceptibility levels between the swine production groups for ciprofloxacin ($p<0.001$) and tetracycline ($p<0.01$) (Table 35). All of the isolates in the nursery production group had decreased susceptibility to ciprofloxacin and tetracycline, and all of the isolates in the grower/finisher group had decreased susceptibility to tetracycline. There was no significant difference in susceptibility among production groups for ampicillin ($p=0.69$), cefoxitin ($p=0.12$), chloramphenical ($p=0.09$), clindamycin ($p=0.29$), imipenem ($p=0.06$), or metronidazole ($p=0.21$). At the time of publication the interpretation of the results for metronidazole and imipenem was under review due to concern in the scientific community concerning the Etest readings at 24 and 48 hour. Thus, in future publications the interpreted binary endpoints may be presented differently than those reported in this publication.

4.4.2 Human binary antimicrobial resistance data

All 271 of the human isolates were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, chloramphenical, and vancomycin (100% susceptibility, 0% resistance). The majority of the human isolates were susceptible to ampicillin (91.5%), clindamycin (87.5%), imipenem (71.2%), metronidazole (86.7%), and tetracycline (95.9%) (Table 36). The majority had decreased susceptibility to cefoxitin (96.3%) and ciprofloxacin (98.5%) (Table 36). There was no significant difference in susceptibility levels between the occupational group cohorts for ampicillin (p=0.51), cefoxitin (p=0.21), ciprofloxacin (p=0.36), clindamycin (p=0.35), imipenem (p=0.89), metronidazole (p=0.28), or tetracycline (p=0.51). At the time of publication the interpretation of the results for metronidazole and imipenem was under review due to concern in the scientific community concerning the Etest readings at 24 and 48 hour. Thus, in future publications the interpreted binary endpoints may be presented differently than those reported in this publication.

Table 34. Prevalence of resistant *Clostridium difficile* swine isolates and 95% confidence interval for the 11 different antimicrobials.

Antimicrobial	Prevalence of resistance (%)	95% Confidence Interval
Amoxicillin-clavulanic acid	0.0	0.0--1.5 *
Ampicillin	15.1	10.9--20.1
Cefoxitin	95.6	92.3--97.8
Chloramphenicol	0.8	0.1--2.8
Clindamycin	44.0	37.8--50.4
Imipenem [1]	58.7	52.4--64.9
Metronidazole [1]	8.3	5.2--12.5
Piperacillin-tazobactam	0.0	0--1.5 *
Tetracycline	2.0	0.6--4.6
Ciprofloxacin	86.5	81.7--90.5
Vancomycin	0.0	0.0--1.5 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

Table 35. Prevalence of resistant *Clostridium difficile* swine isolates and 95% confidence interval among swine production groups for the 11 different antimicrobials.

	farrowing (n=175)		grower/finisher (n=37)		nursery (n=14)		breeding (n=26)	
Antimicrobial	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI
Amoxicillin-clavulanic acid	0.0	0.0--2.1 *	0.0	0.0--9.5 *	0.0	0.0--23.2 *	0.0	0.0--13.2 *
Ampicillin	14.3	9.5--20.4	18.9	8.0--35.2	7.1	0.2--33.9	19.2	6.6--39.4
Cefoxitin	96.6	92.7--98.7	89.2	74.6--97.0	92.9	66.1--99.8	100.0	86.8--100 *
Chloramphenicol	0.0	0.0--2.1 *	2.7	0.1--14.2	0.0	0.0--23.2 *	3.8	0.1--19.6
Clindamycin	46.3	38.7--54.0	29.7	15.9--47.0	50.0	23.0--77.0	46.2	26.6--66.6
Imipenem [1]	62.9	55.2--70.0	45.9	29.5--63.1	71.4	41.9--91.6	42.3	23.4--63.1
Metronidazole [1]	6.3	3.2--11.0	13.5	4.5--28.8	14.3	1.8--42.8	11.5	2.4--30.2
Piperacillin-tazobactam	0.0	0.0--2.1 *	0.0	0.0--9.5 *	0.0	0.0--23.2 *	0.0	0.0--13.2 *
Tetracycline	0.6	0.01--3.1	0.0	0.0--9.5 *	0.0	0.0--23.2 *	15.4	4.4--34.9
Ciprofloxacin	89.1	83.6--93.3	89.2	74.6--97.0	100.0	76.8--100 *	57.7	36.9--76.6
Vancomycin	0.0	0.0--2.1 *	0.0	0.0--9.5 *	0.0	0.0--23.2 *	0.0	0.0--13.2 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

Table 36. Prevalence of resistant *Clostridium difficile* human isolates and 95% confidence interval for the 11 different antimicrobials.

Antimicrobial	Prevalence of resistance (%)	95% Confidence Interval
Amoxicillin-clavulanic acid	0.0	0.0--1.4 *
Ampicillin	8.5	5.5--12.5
Cefoxitin	95.9	92.9--98.0
Chloramphenicol	0.0	0.0--1.4 *
Clindamycin	12.5	8.8--17.1
Imipenem [1]	28.8	23.5--34.6
Metronidazole [1]	13.3	9.5--17.9
Piperacillin-tazobactam	0.0	0.0--1.4 *
Tetracycline	4.1	2.1--7.2
Ciprofloxacin	98.2	95.7--99.4
Vancomycin	0.0	0.0--1.4 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

4.4.3 Comparison of binary antimicrobial resistance data across host species

All isolates in both the swine and human populations were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, and vancomycin (100% susceptibility, 0% resistance). A majority of the isolates were susceptible to ampicillin (88.5%), chloramphenical (99.6%), clindamycin (72.5%), imipenem (57.0%), metronidazole (89.3%), and tetracycline (96.9%) (Table 37). The majority of the isolates had decreased susceptiblity to cefoxitin (96.0%) and ciprofloxacin (92.7%) (Table 37). In unconditional analyses (unadjusted for potential confounders), when comparing susceptibility between host species, there was significantly ($p<0.05$) decreased susceptiblity to ampicillin, clindamycin, and imipenem among the swine isolates and there was significantly ($p<0.05$) descreased susceptiblity to ciprofloxacin among the human isolates (Table 38). There was a significant difference in susceptibility levels among swine production group/human occupational group cohorts for chloramphenical ($p<0.05$), ciprofloxacin ($p<0.001$), clindamycin ($p<0.001$), imipenem ($p<0.001$), and tetracycline ($p<0.001$) (Table 39). There was a higher prevalence of decreased susceptibility to chloramphenical in the older swine production groups when compared to the younger swine production groups and human occupational group cohorts.

A higher prevalence of decreased susceptibility for imipenem was found in the younger swine production groups than in the older swine production groups and human occupational group cohorts. Interestingly, the highest percentage of decreased susceptibility to tetracycline was found in the swine breeding production group. There was also a significant difference in susceptibility levels between seasons for clindamycin ($p<0.05$), imipenem ($p<0.001$), and tetracycline ($p<0.001$) (Table 40). The highest percentage of decreased susceptibility for clindamycin and imipenem was found in the winter and the highest for tetracycline was found in the spring, although decreased susceptibility was low in four seasons. At the time of publication the interpretation of the results for metronidazole and imipenem was under review due to concern in the scientific community concerning the Etest readings at 24 and 48 hour. Thus, in future publications the interpreted binary endpoints may be presented differently than those reported in this publication.

Table 37. Prevalence of resistant *Clostridum difficile* isolates and 95% confidence interval for all *C. difficile* isolates tested across both host species and across all production groups/cohorts for the 11 different antimicrobials.

Antimicrobial	Prev of resistance (%)	95% CI
Amoxicillin / clavulanic acid (2:1)	0.0	0.0--1.1 *
Ampicillin	11.5	8.9--14.5
Cefoxitin	96.0	93.9--97.5
Chloramphenical	0.4	0.05--1.4
Ciprofloxacin	92.7	90.2--94.8
Clindamycin	27.5	23.7--31.6
Imipenen [1]	43.0	38.7--47.4
Metronidazole [1]	10.7	8.2--13.7
Piperacillin / tazobactam	0.0	0.0--1.1 *
Tetracycline	3.1	1.8--4.9
Vancomycin	0.0	0.0--1.1 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

Table 38. Prevalence of resistant *Clostridium difficile* isolates and 95% confidence interval for *C. difficile* isolates compared between host species for the 11 different antimicrobials.

	Swine		Human	
Antimicrobial	Prev of resistance (%)	95% CI	Prev of resistance (%)	95% CI
Amoxicillin-clavulanic acid	0.0	0.0--1.5 *	0.0	0.0--1.4 *
Ampicillin	15.1	10.9--20.1	8.5	5.5--12.5
Cefoxitin	95.6	92.3--97.8	95.9	92.9--98.0
Chloramphenicol	0.8	0.1--2.8	0.0	0.0--1.4 *
Clindamycin	44.0	37.8--50.4	12.5	8.8--17.1
Imipenem [1]	58.7	52.4--64.9	28.8	23.5--34.6
Metronidazole [1]	8.3	5.2--12.5	13.3	9.5--17.9
Piperacillin-tazobactam	0.0	0--1.5 *	0.0	0.0--1.4 *
Tetracycline	2.0	0.6--4.6	4.1	2.1--7.2
Ciprofloxacin	86.5	81.7--90.5	98.2	95.7--99.4
Vancomycin	0.0	0.0--1.5 *	0.0	0.0--1.4 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

Table 39. Prevalence of resistant *Clostridium difficile* isolates and 95% confidence interval for *C. difficile* isolates compared across production group/occupational group cohorts for both host species for the 11 different antimicrobials.

	farrowing (n=175)		grower/finisher (n=37)		nursery (n=14)		breeding (n=26)		non-worker (n=140)		worker (n=131)	
Antimicrobial	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI
Amoxicillin-clavulanic acid	0.0	0.0--2.1 *	0.0	0.0--9.5 *	0.0	0.0--23.2 *	0.0	0.0--13.2 *	0.0	0.0--2.6 *	0	0.0--2.8 *
Ampicillin	14.3	9.5--20.4	18.9	8.0--35.2	7.1	0.2--33.9	19.2	6.6--39.4	10.0	5.6--16.2	6.9	3.2--12.6
Cefoxitin	96.6	92.7--98.7	89.2	74.6--97.0	92.9	66.1--99.8	100.0	86.8--100 *	97.1	92.8--99.2	94.7	89.3--97.8
Chloramphenicol	0.0	0.0--2.1 *	2.7	0.1--14.2	0.0	0.0--23.2 *	3.8	0.1--19.6	0.0	0.0--2.6 *	0	0.0--2.8 *
Clindamycin	46.3	38.7--54.0	29.7	15.9--47.0	50.0	23.0--77.0	46.2	26.6--66.6	15.0	9.5--22.0	9.9	5.4--16.4
Imipenem [1]	62.9	55.2--70.0	45.9	29.5--63.1	71.4	41.9--91.6	42.3	23.4--63.1	28.6	21.3--36.8	29	21.4--37.6
Metronidazole [1]	6.3	3.2--11.0	13.5	4.5--28.8	14.3	1.8--42.8	11.5	2.4--30.2	11.4	6.7--17.9	15.3	9.6--22.6
Piperacillin-tazobactam	0.0	0.0--2.1 *	0.0	0.0--9.5 *	0.0	0.0--23.2 *	0.0	0.0--13.2 *	0.0	0.0--2.6 *	0	0.0--2.8 *
Tetracycline	0.6	0.01--3.1	0.0	0.0--9.5 *	0.0	0.0--23.2 *	15.4	4.4--34.9	6.5	3.0--11.9	1.5	0.2--5.4
Ciprofloxacin	89.1	83.6--93.3	89.2	74.6--97.0	100.0	76.8--100 *	57.7	36.9--76.6	98.6	94.9--99.8	97.7	93.5--99.5
Vancomycin	0.0	0.0--2.1 *	0.0	0.0--9.5 *	0.0	0.0--23.2 *	0.0	0.0--13.2 *	0.0	0.0--2.6 *	0	0.0--2.8 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

Table 40. Prevalence of resistant *Clostridium difficile* isolates and 95% confidence interval for *C. difficile* isolates compared across seasons for both host species for the 11 different antimicrobials.

	winter (n=122)		spring (n=161)		summer (n=119)		fall (n=121)	
Antimicrobial	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI	Prev of res (%)	95% CI
Amoxicillin-clavulanic acid	0.0	0.0--3.0 *	0.0	0.0--2.3 *	0.0	0.0--3.1 *	0.0	0.0--3.0 *
Ampicillin	9.0	4.6--15.6	12.4	7.8--18.5	12.6	7.2--19.9	12.4	7.1--19.6
Cefoxitin	96.7	91.8--99.1	97.5	93.8--99.3	94.1	88.3--97.6	94.2	88.4--97.6
Chloramphenicol	0.0	0.0--3.0 *	1.2	0.2--4.4	0.0	0.0--3.1 *	0.0	0.0--3.0 *
Clindamycin	36.1	27.6--45.3	26.7	20.1--34.2	19.3	12.7--27.6	28.9	21.0--37.9
Imipenem [1]	55.7	46.5--64.7	46.6	38.7--54.6	35.3	26.8--44.6	33.9	25.5--43.0
Metronidazole [1]	17.2	11.0--25.1	8.1	4.4--13.4	9.2	4.7--15.9	9.9	5.2--16.7
Piperacillin-tazobactam	0.0	0.0--3.0 *	0.0	0.0--2.3 *	0.0	0.0--3.1 *	0.0	0.0--3.0 *
Tetracycline	1.6	0.2--5.8	3.8	1.4--8.0	4.2	1.4--9.5	2.5	0.5--7.1
Ciprofloxacin	94.3	88.5--97.7	93.2	88.1--96.5	89.9	83.0--94.7	92.6	86.3--96.5
Vancomycin	0.0	0.0--3.0 *	0.0	0.0--2.3 *	0.0	0.0--3.1 *	0.0	0.0--3.0 *

* one-sided 97.5% confidence interval

[1] interpretation of the results is still under review

4.4.4 Within and between host species differences in C. difficile *minimum inhibitory concentrations (MIC) for 11 antimicrobials*

Non-parametric survival analysis was used to assess differences in the distribution of MIC values both within and between the host species. Figures 16 through 26 show the distribution of MIC values for the 11 different antimicrobials tested for all 523 *C. difficile* isolates (i.e., across host species). Obvious differences can be seen in the distribution of MIC values between the host species suggesting that a common parametric approach to these data would be inappropriate (Figures 16-26). Non-parametric approaches are robust to these differences. Significant ($p<0.05$) differences (based on log-rank test) were found among host species with trends towards higher MIC values for imipenem, ampicillin, and clindamycin in the swine isolates compared to the human isolates (Figures 27-29). Significant ($p<0.05$) trends towards higher MIC values for cefoxitin, ciprofloxacin, amoxicillin/clavulanic acid, piperacillin/tazobactam, metronidazole, and tetracycline were found in the human isolates compared to the swine isolates (Figures 30-35).

Key MIC values were expressed graphically as MIC_{50} and MIC_{90} respectively. A significant ($p<0.05$) difference was found among swine production groups for MIC's for amoxicillin/clavulanic acid and clindamycin (Figures 36 and 37). Reduced susceptibility to amoxicillin/clavulanic acid was found in the grower/finisher pigs and reduced susceptibility to clindamycin among pigs in the farrowing barn. No significant differences ($P > 0.05$) were found in the resistance patterns of isolates arising from the different human occupational group cohorts. At the time of publication the interpretation of the results for metronidazole and imipenem was under review due to concern in the scientific community concerning the Etest readings at 24 and 48 hour. Thus, in future publications the interpreted binary endpoints may be presented differently than those reported in this publication.

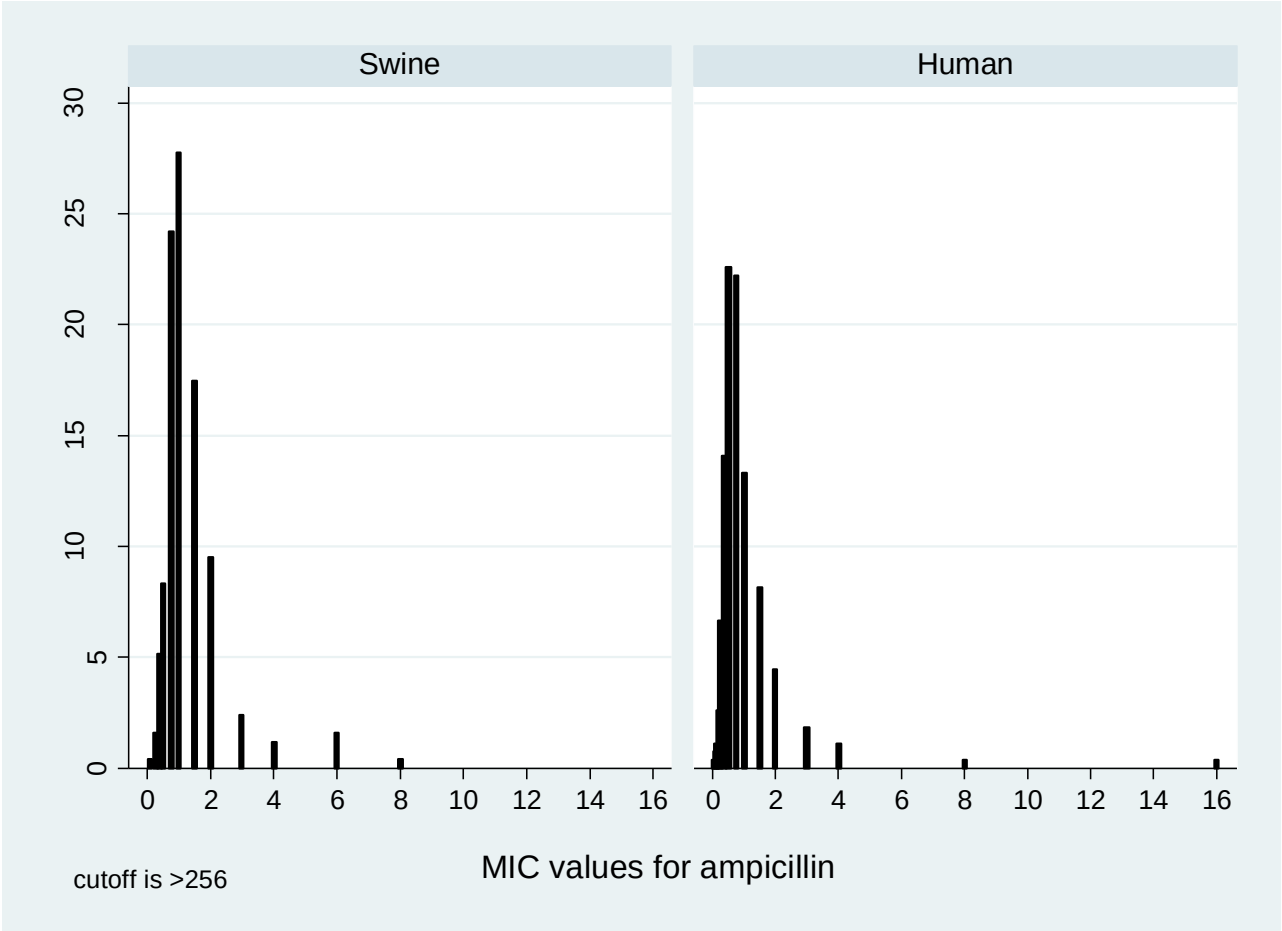

Figure 16. Distribution of MIC values for ampicillin among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤0.5, Resistant≥2).

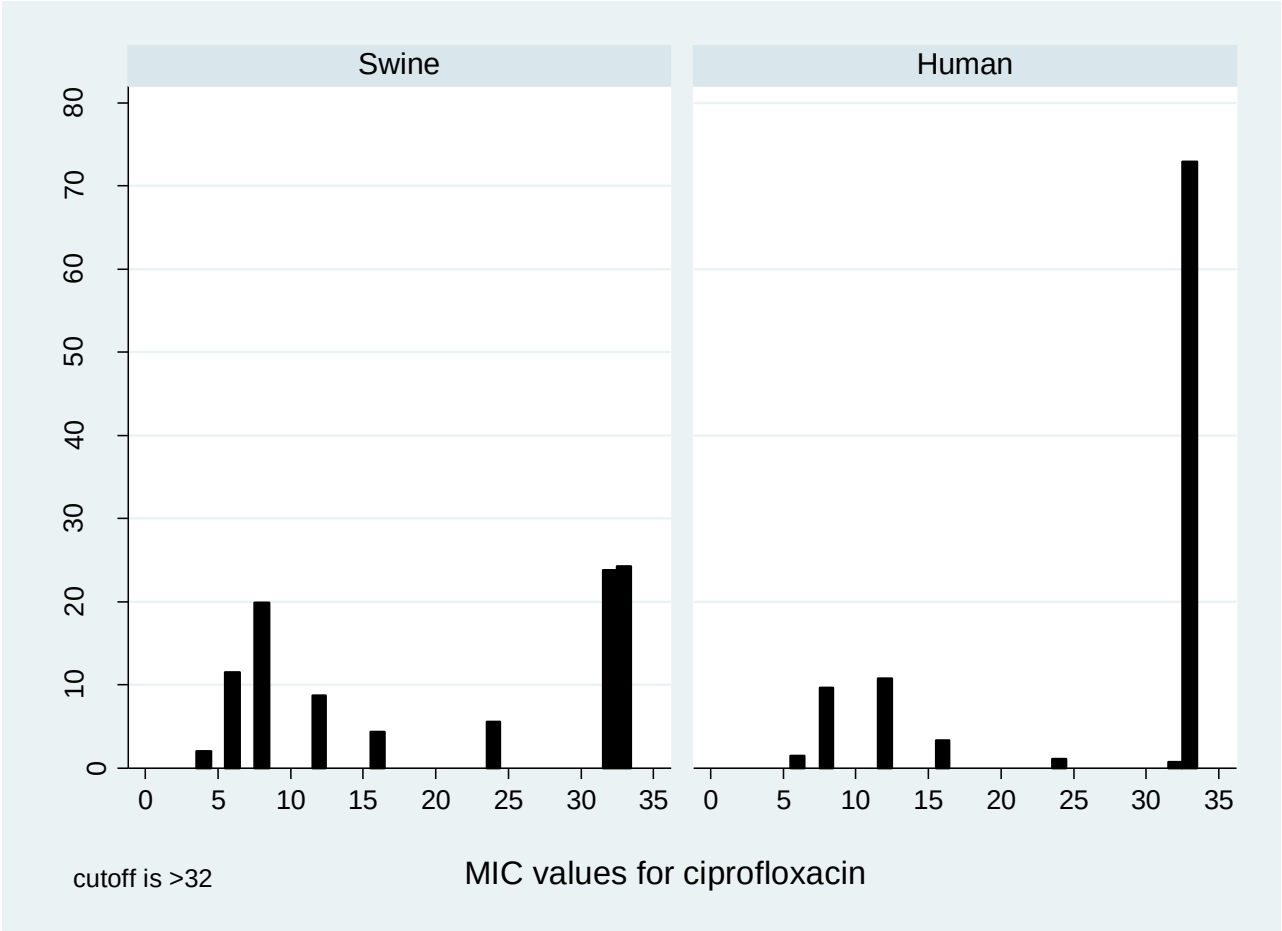

Figure 17. Distribution of MIC values for ciprofloxacin among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤2, Resistant≥8).

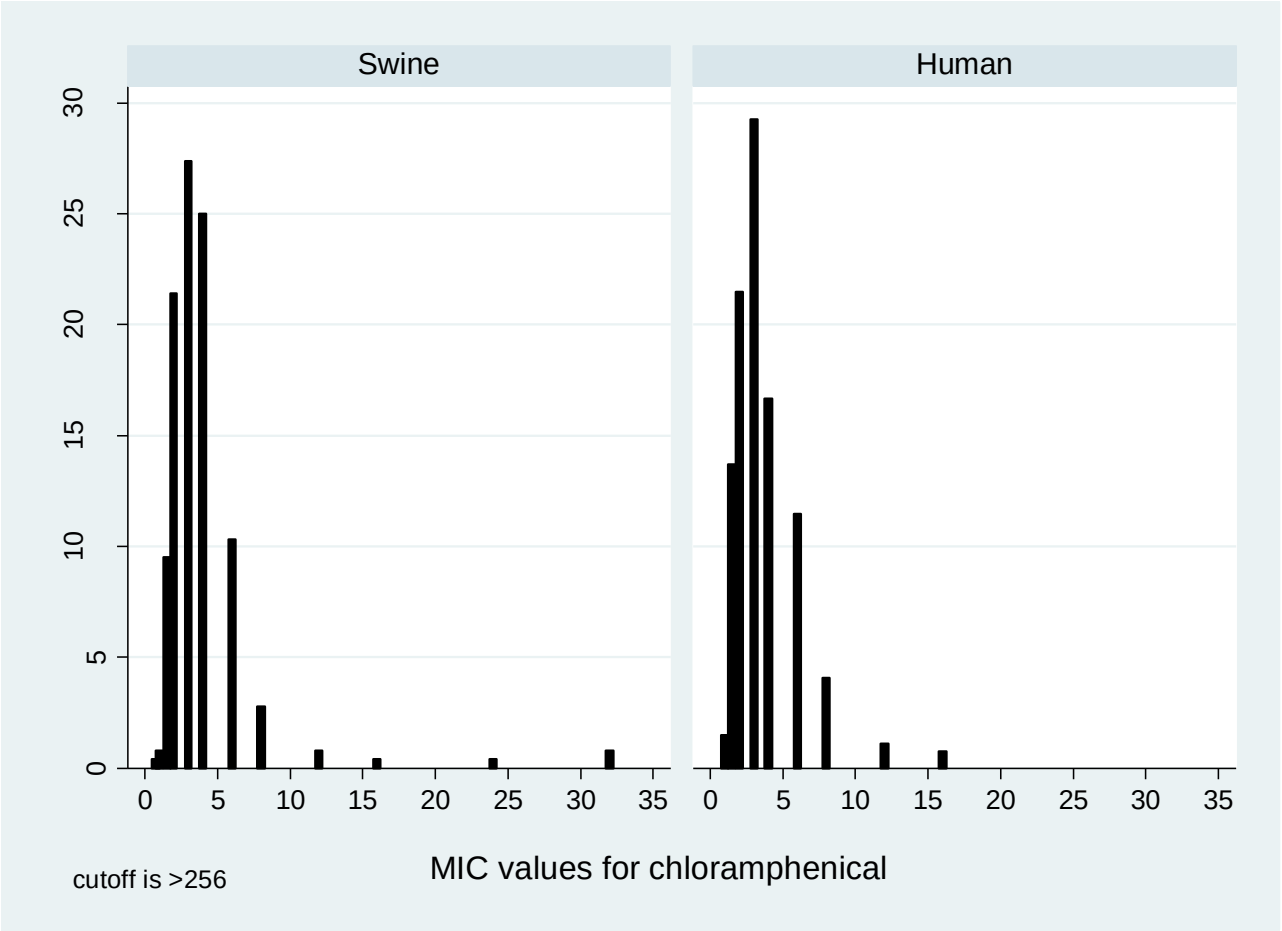

Figure 18. Distribution of MIC values for chloramphenical among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤8, Resistant≥32).

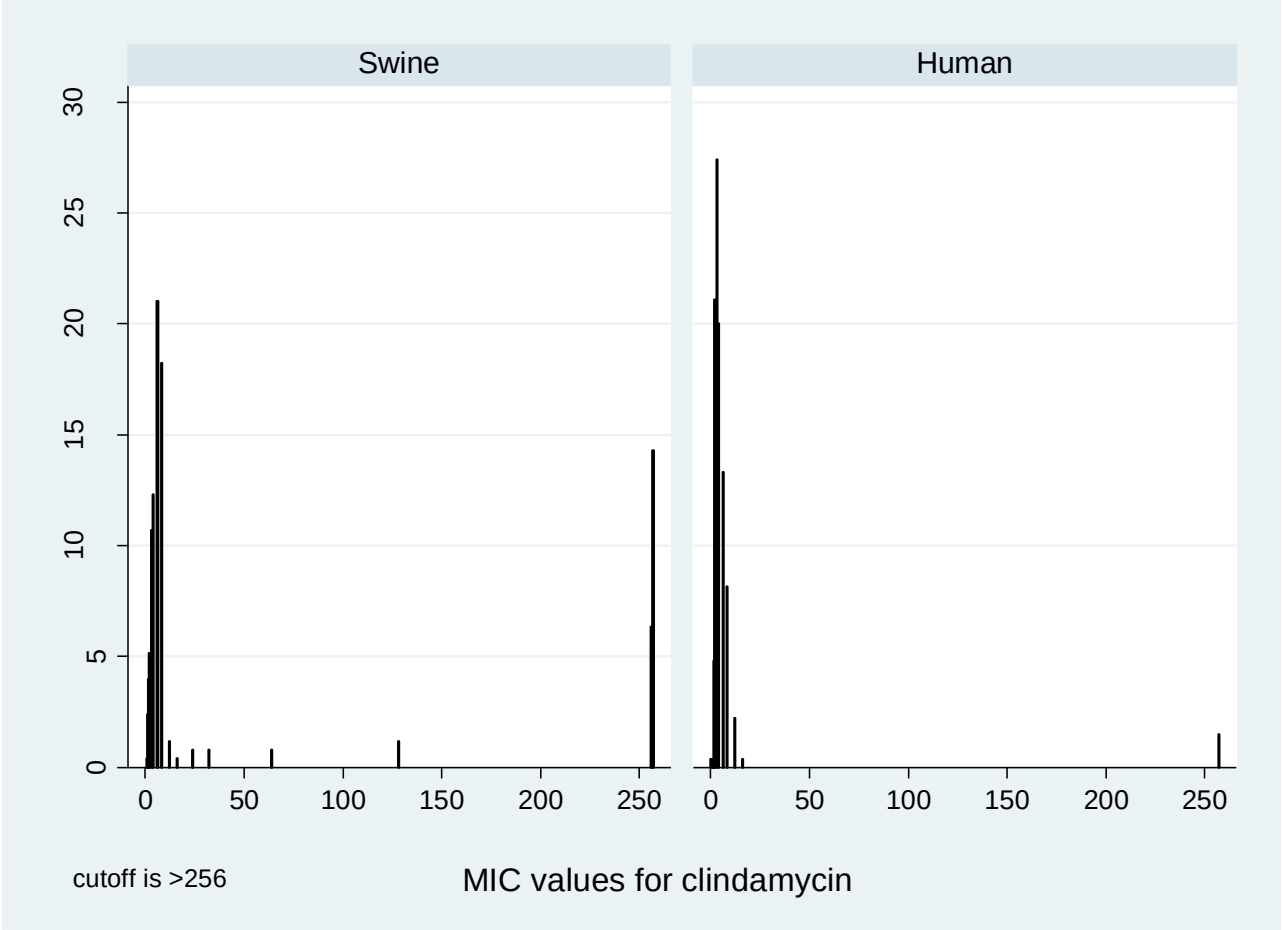

Figure 19. Distribution of MIC values for clindamycin among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤2, Resistant≥8).

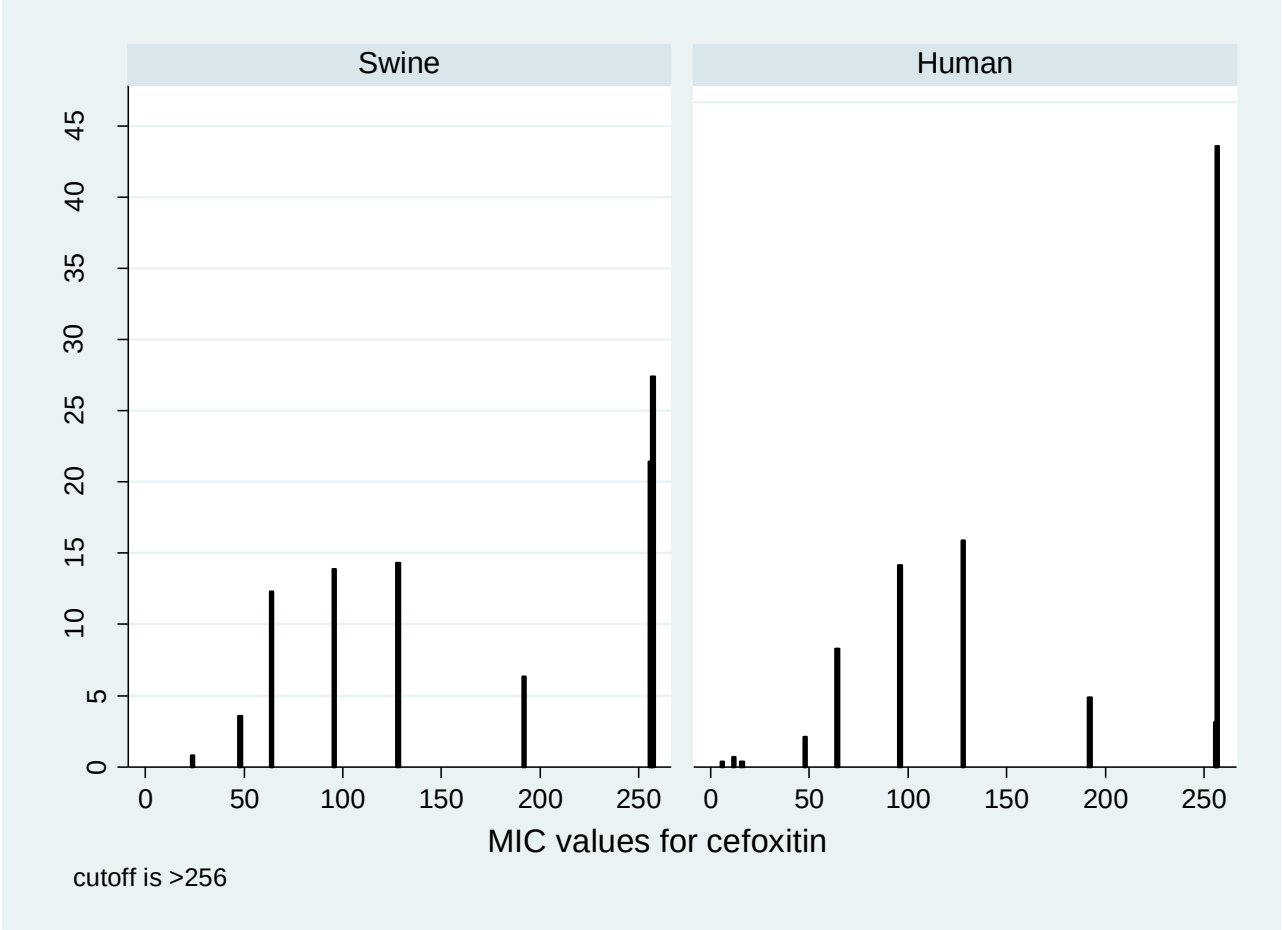

Figure 20. Distribution of MIC values for cefoxitin among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤16, Resistant≥64).

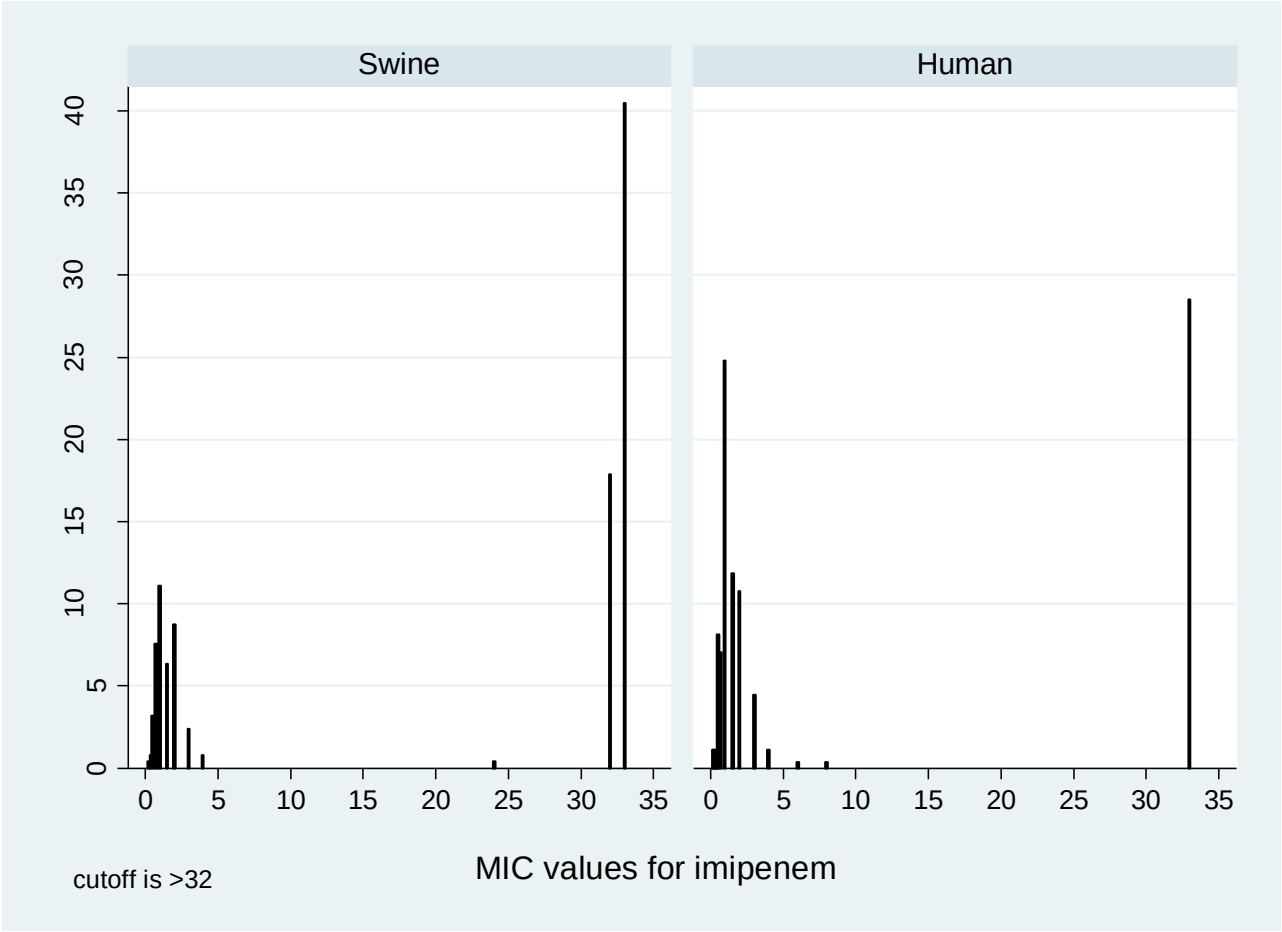

Figure 21. Distribution of MIC values for imipenem among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤4, Resistant≥16). Interpretation of results under review.

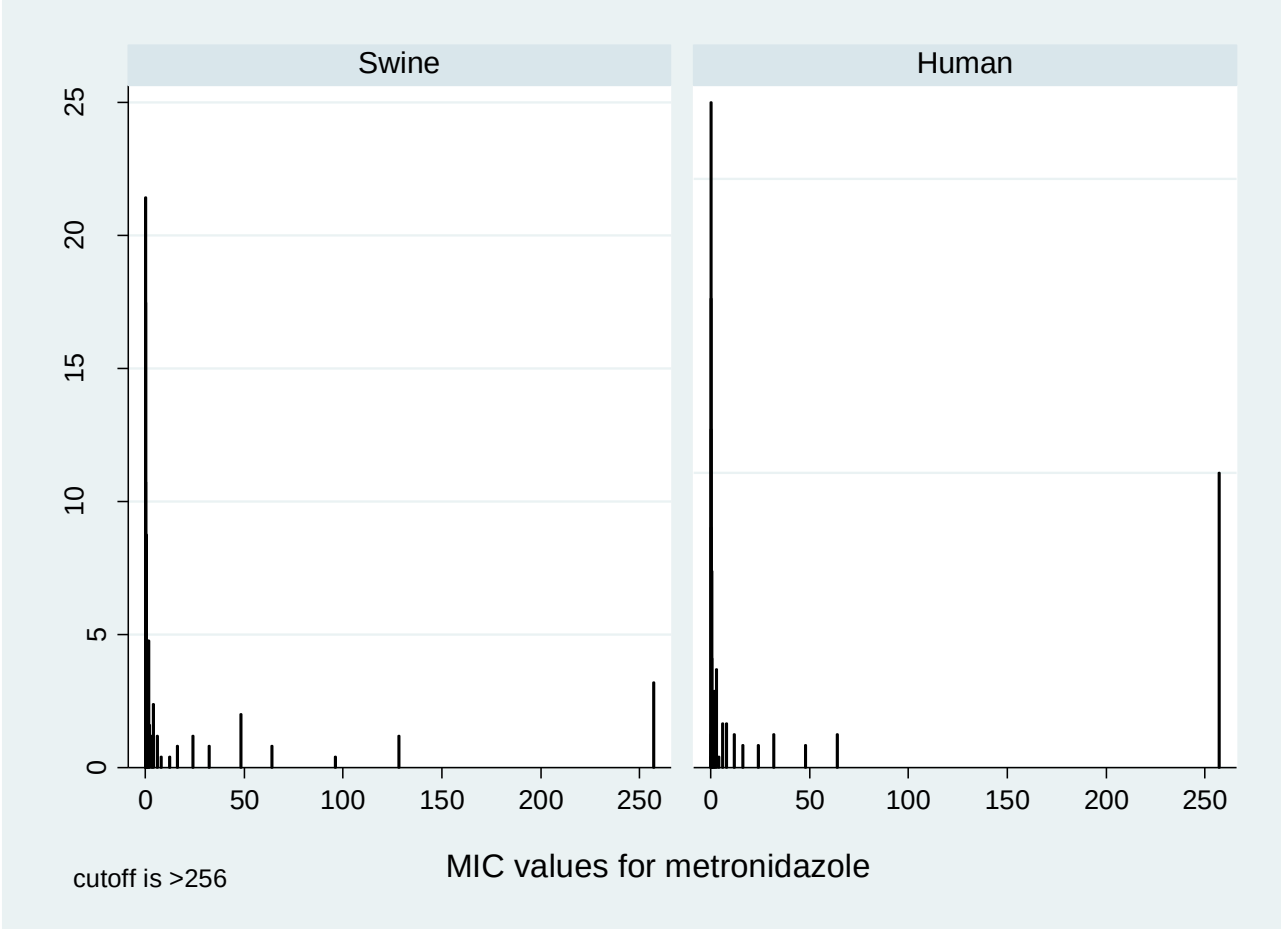

Figure 22. Distribution of MIC values for metronidazole among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤8, Resistant≥32). Interpretation of results is under review.

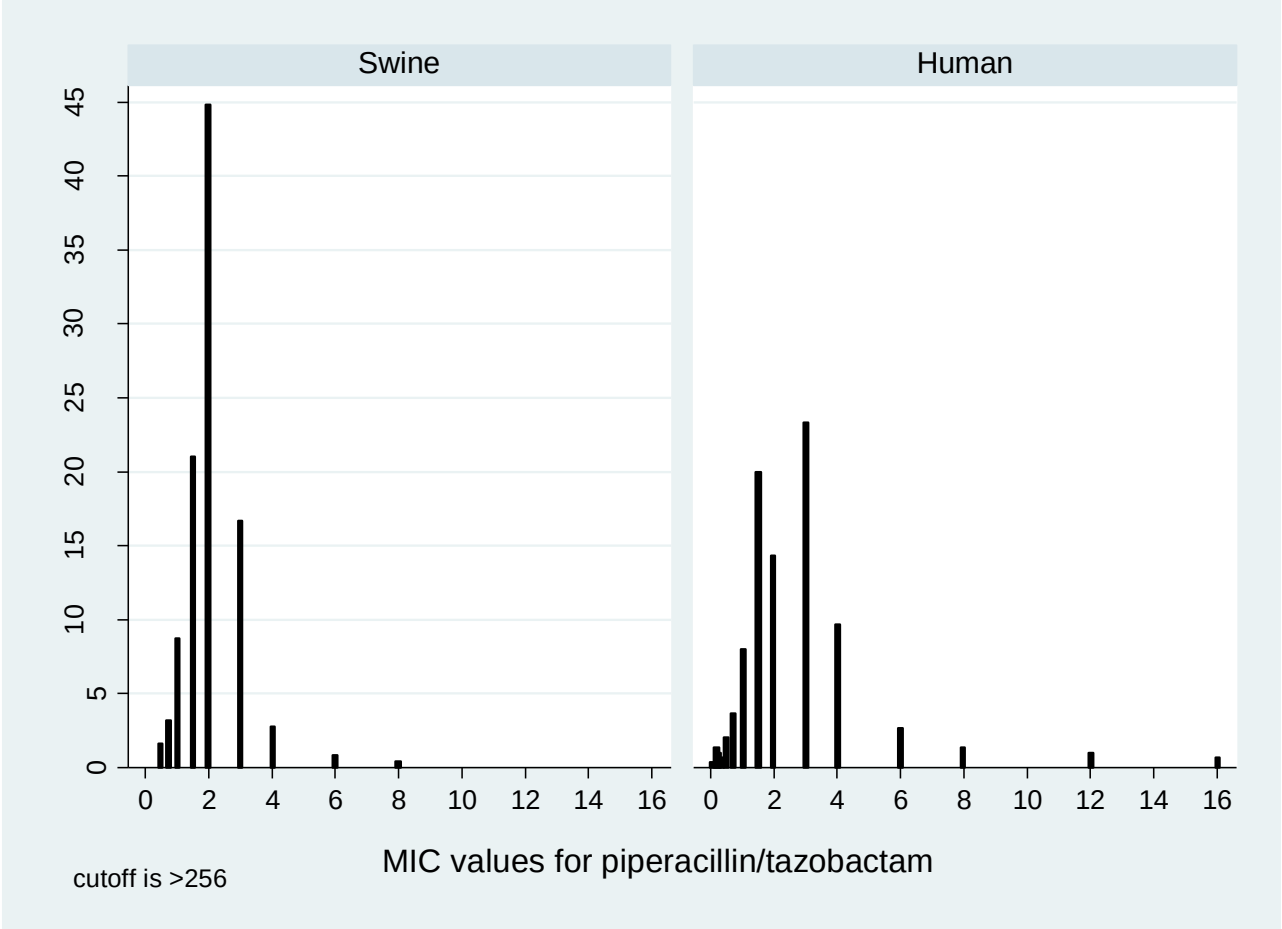

Figure 23. Distribution of MIC values for piperacillin/tazobactam among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤32, Resistant≥128).

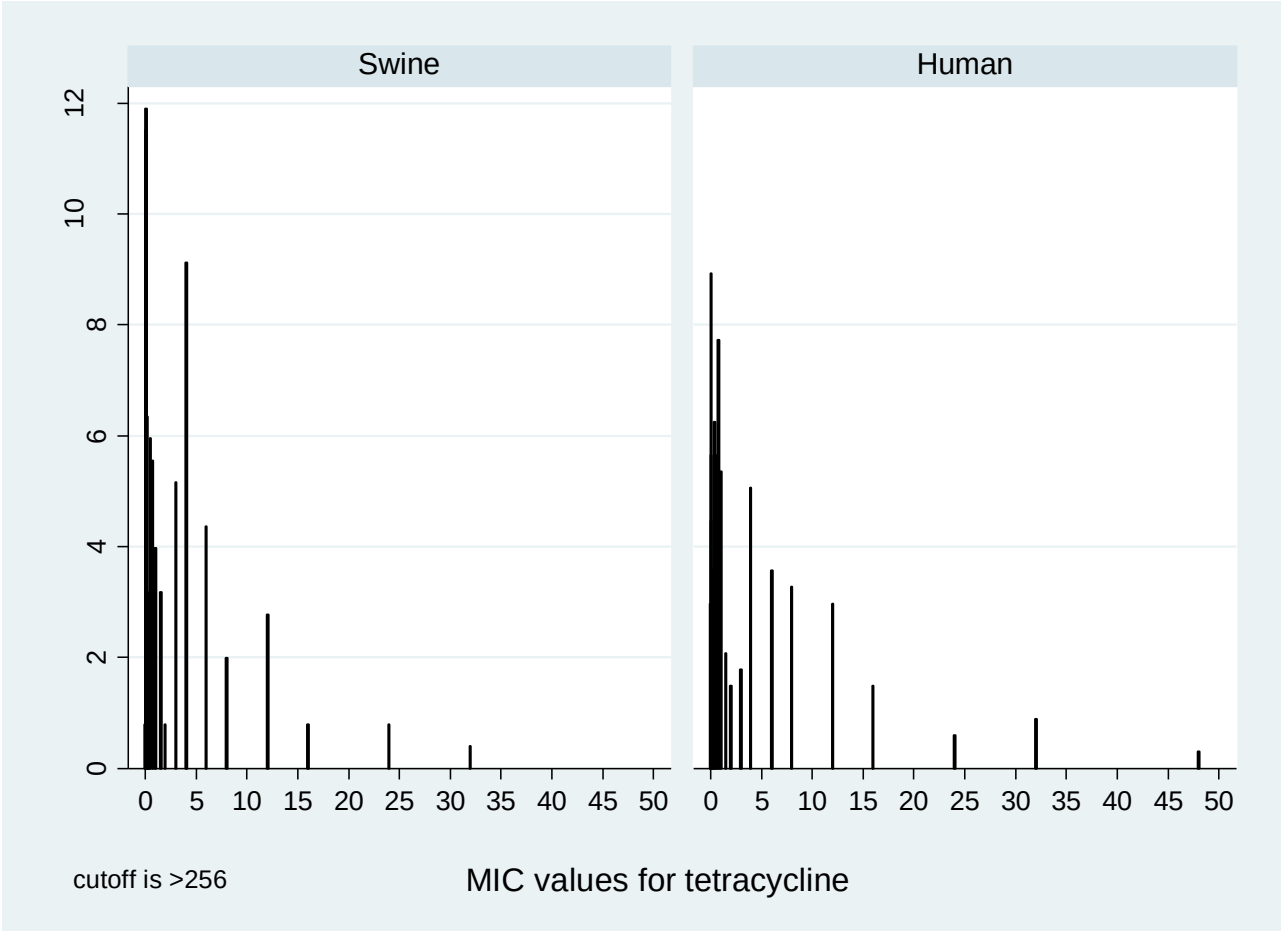

Figure 24. Distribution of MIC values for tetracycline among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤4, Resistant≥16).

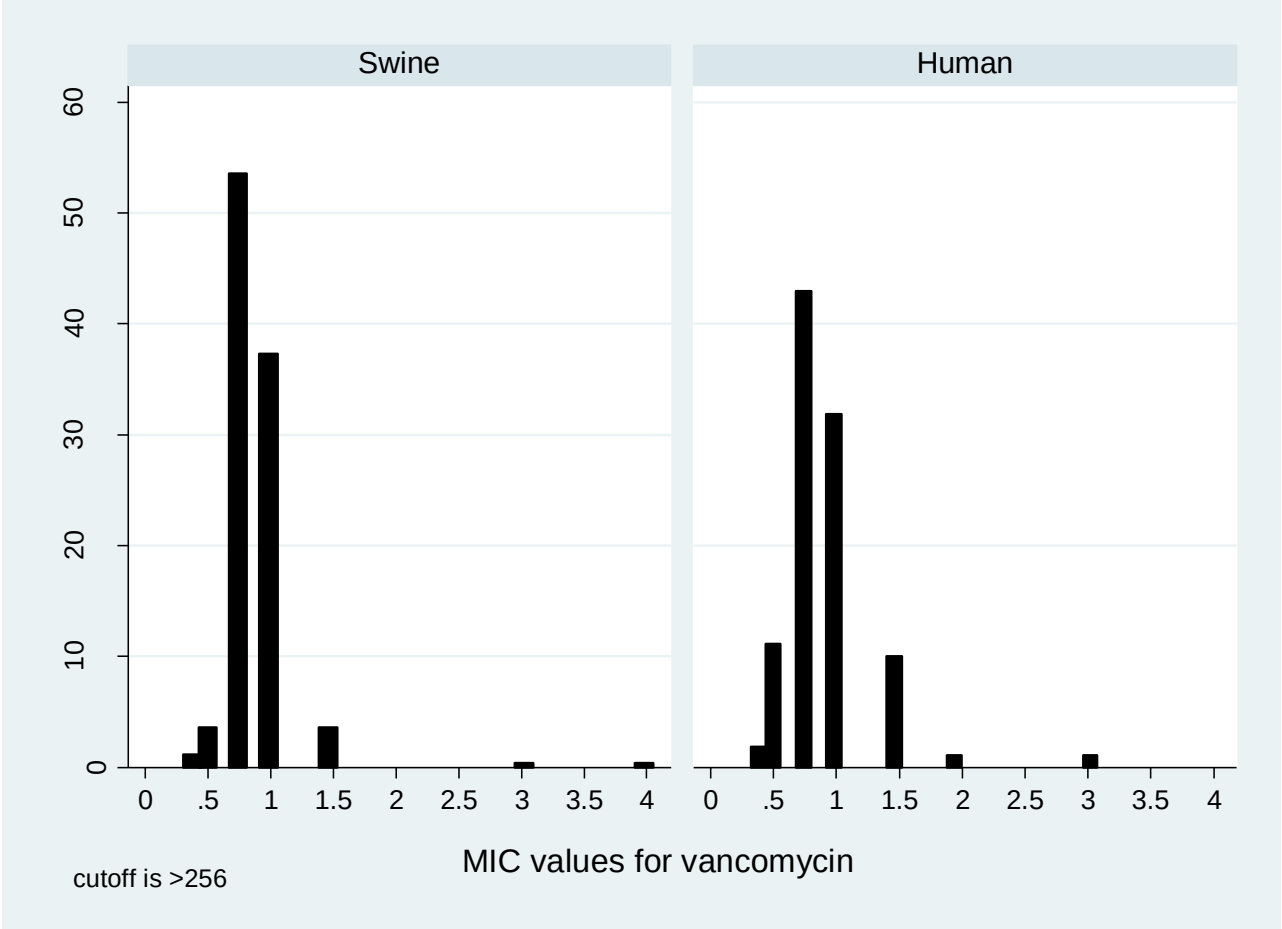

Figure 25. Distribution of MIC values for vancomycin among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤4, Resistant≥32).

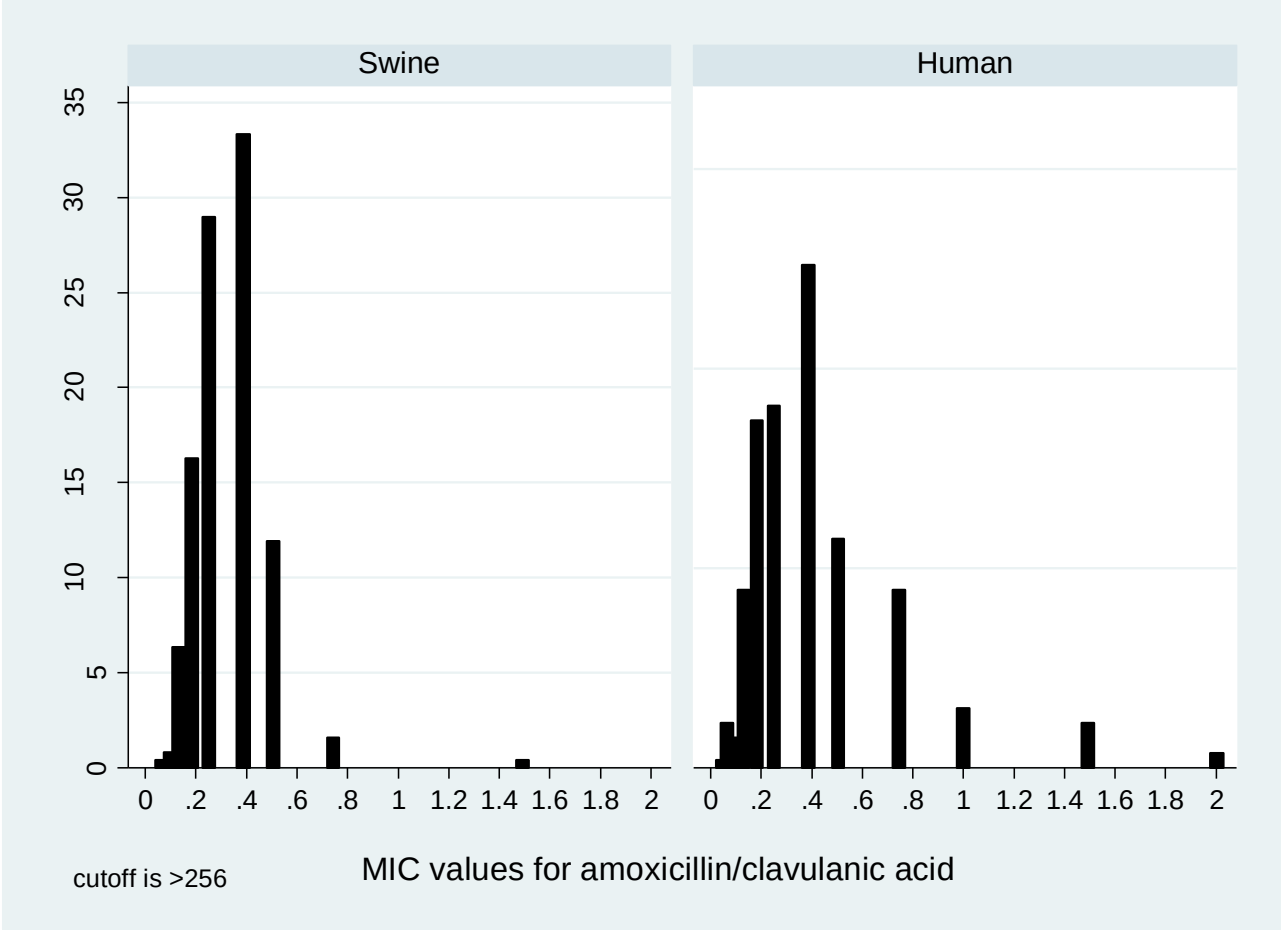

Figure 26. Distribution of MIC values for amoxicillin/clavulanic acid among *Clostridium difficile* isolated from human (n=271) and swine (n=252); MIC cutoff values (Susceptible≤4, Resistant≥16).

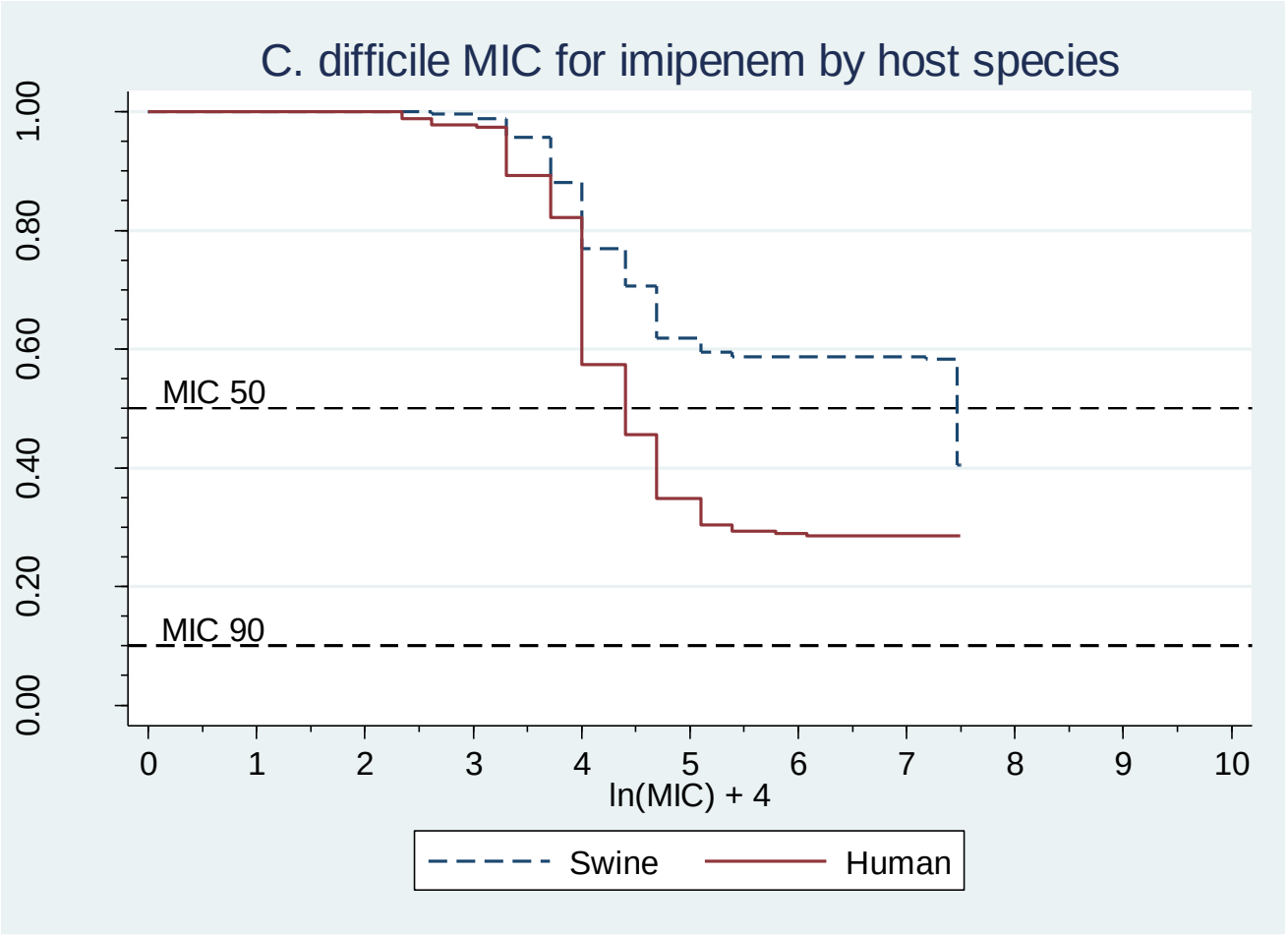

Figure 27. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for imipenem by host species ($p<0.001$; log-rank test).

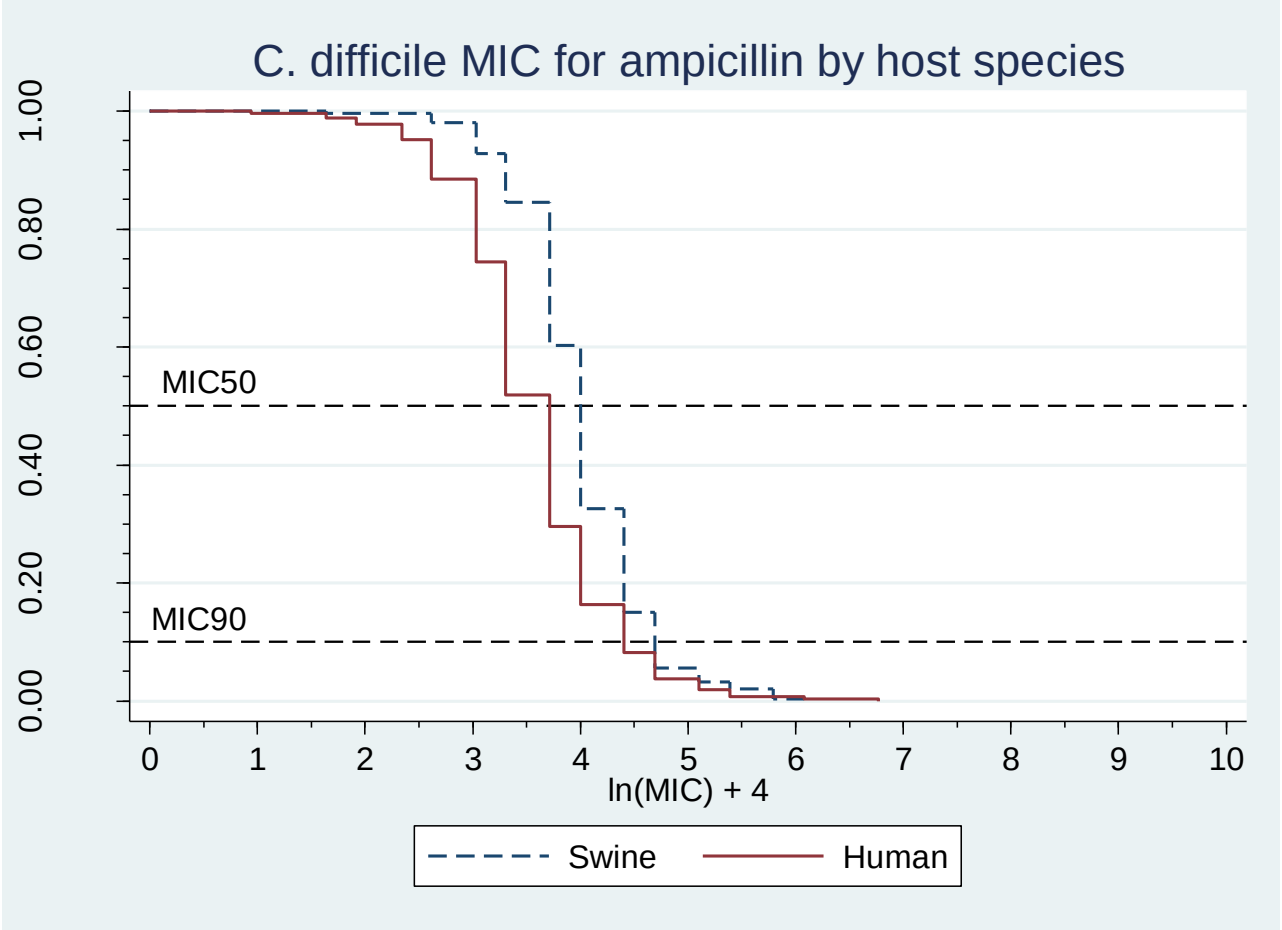

Figure 28. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for ampicillin by host species ($p<0.001$; log-rank test).

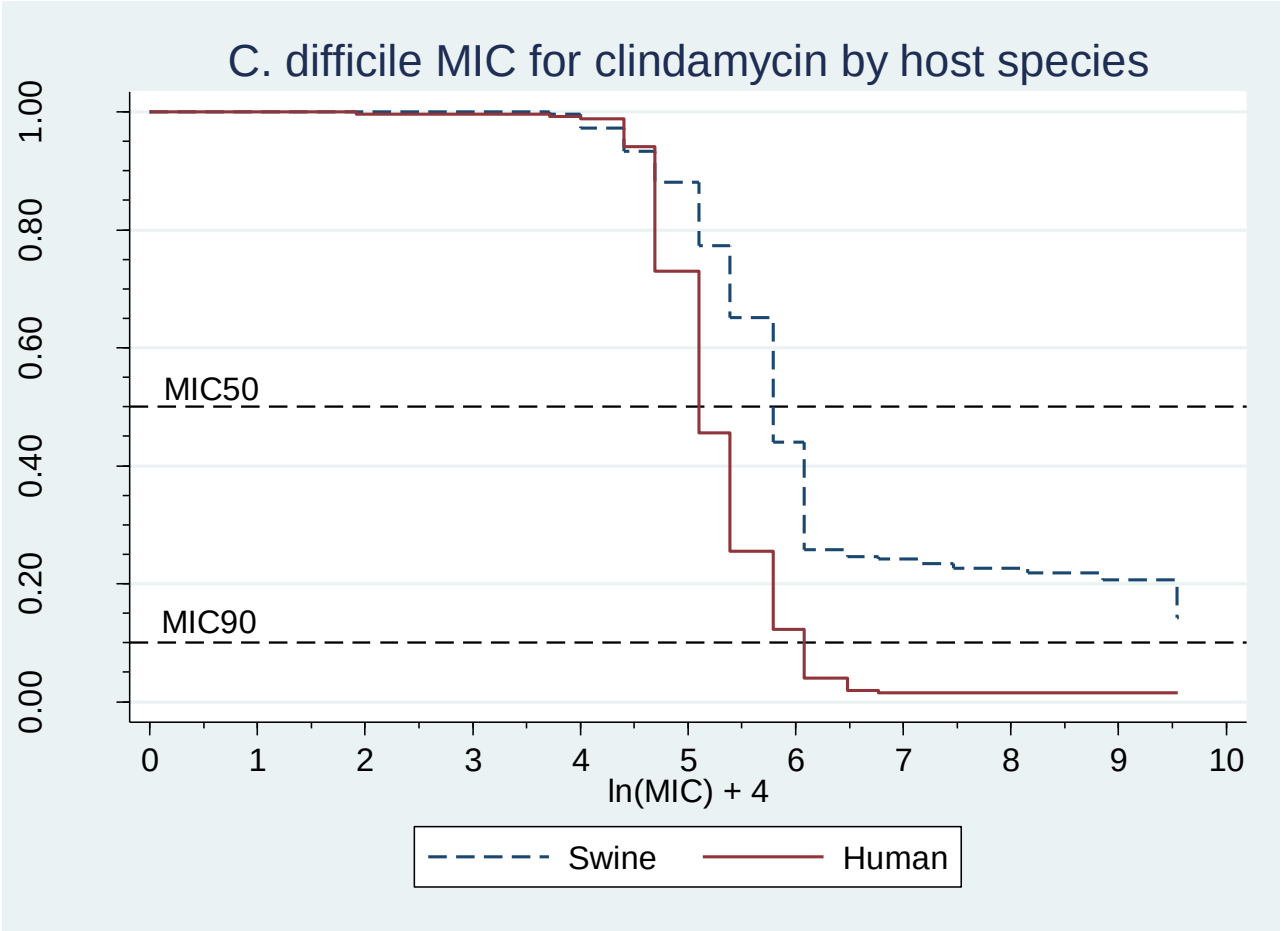

Figure 29. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for clindamycin by host species ($p<0.001$; log-rank test).

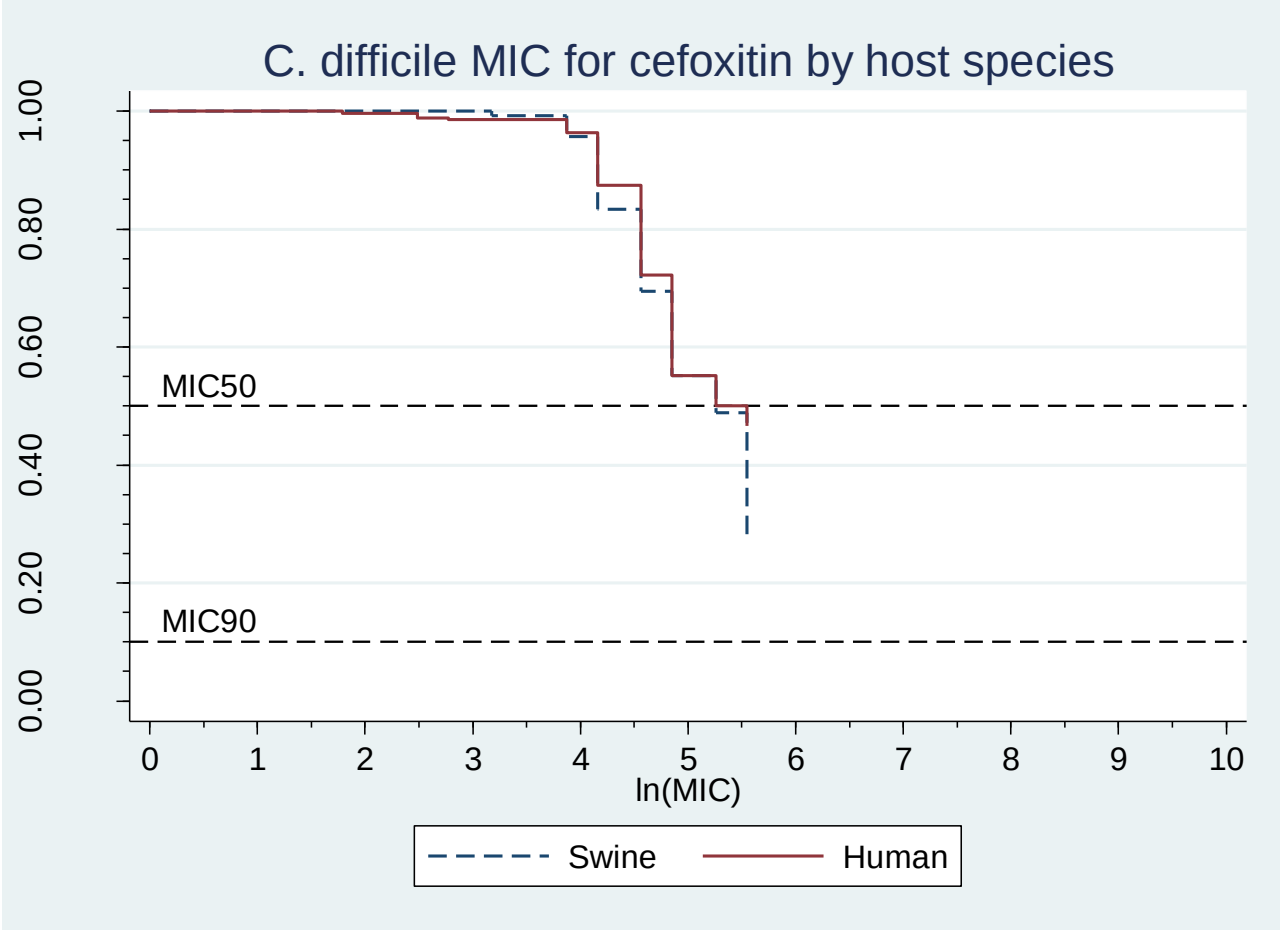

Figure 30. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for cefoxitin by host species (p=0.001; log-rank test).

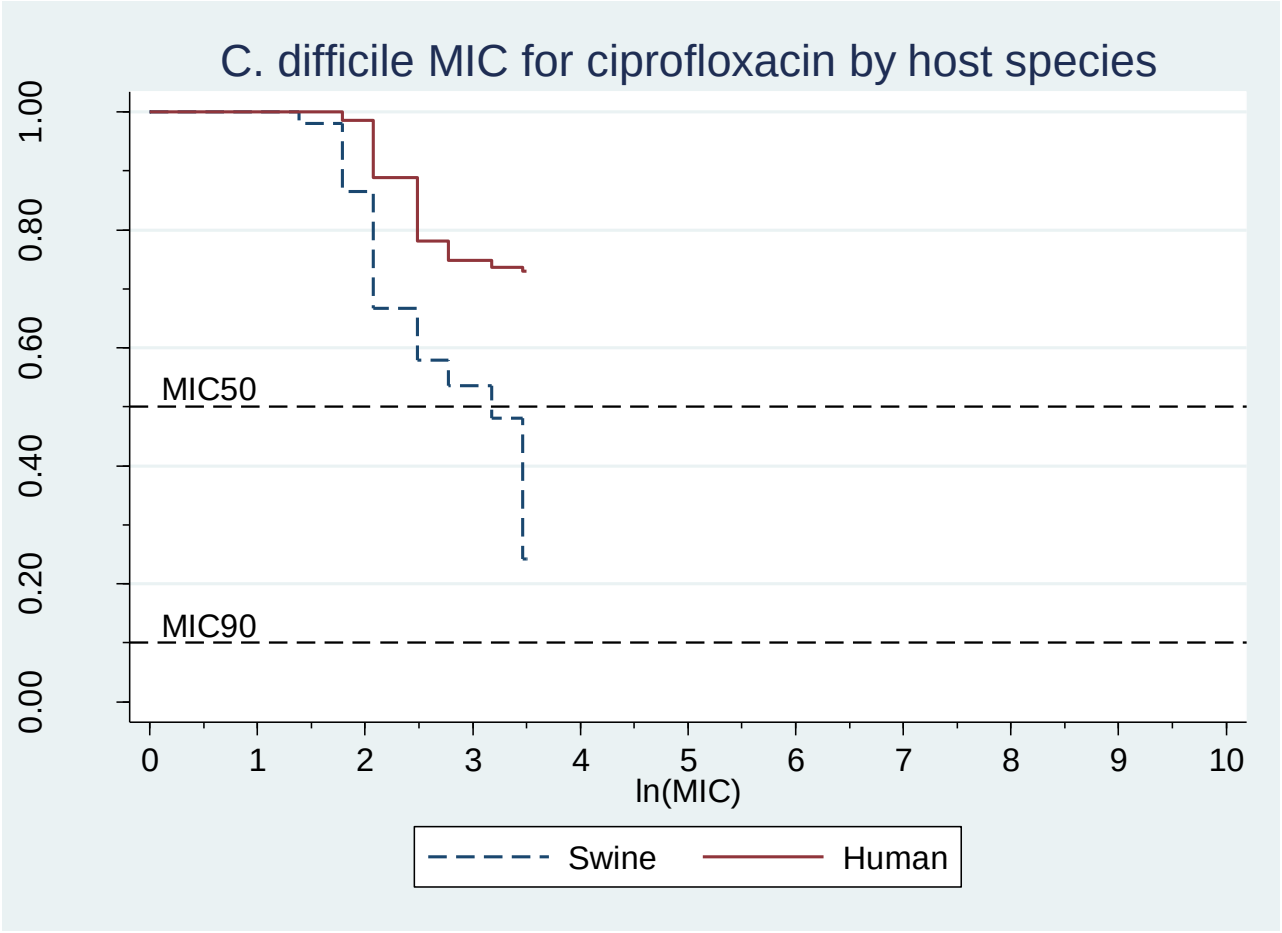

Figure 31. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for ciprofloxacin by host species ($p<0.001$; log-rank test).

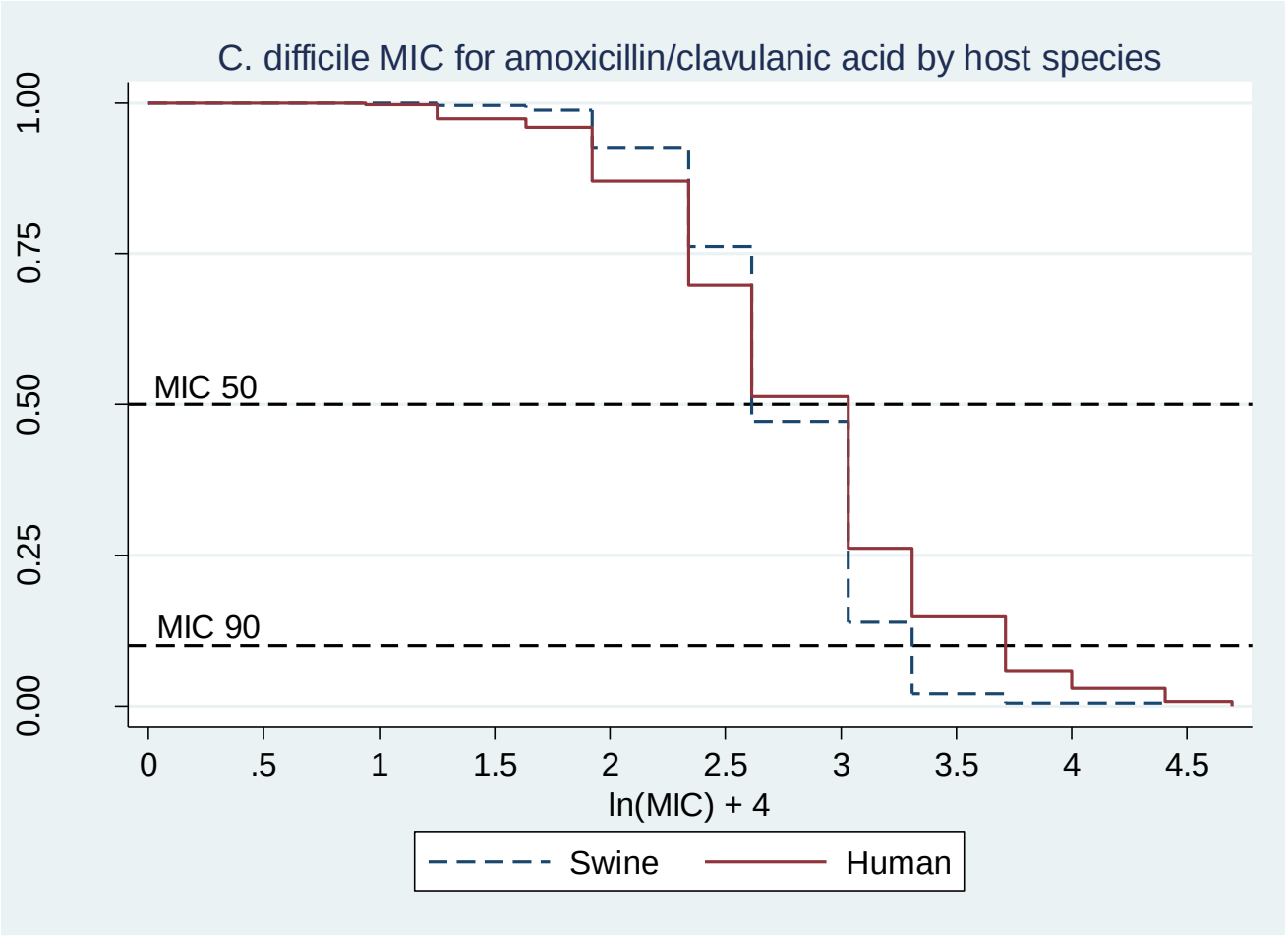

Figure 32. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for amoxicillin/clavulanic acid by host species (p=0.003; log-rank test).

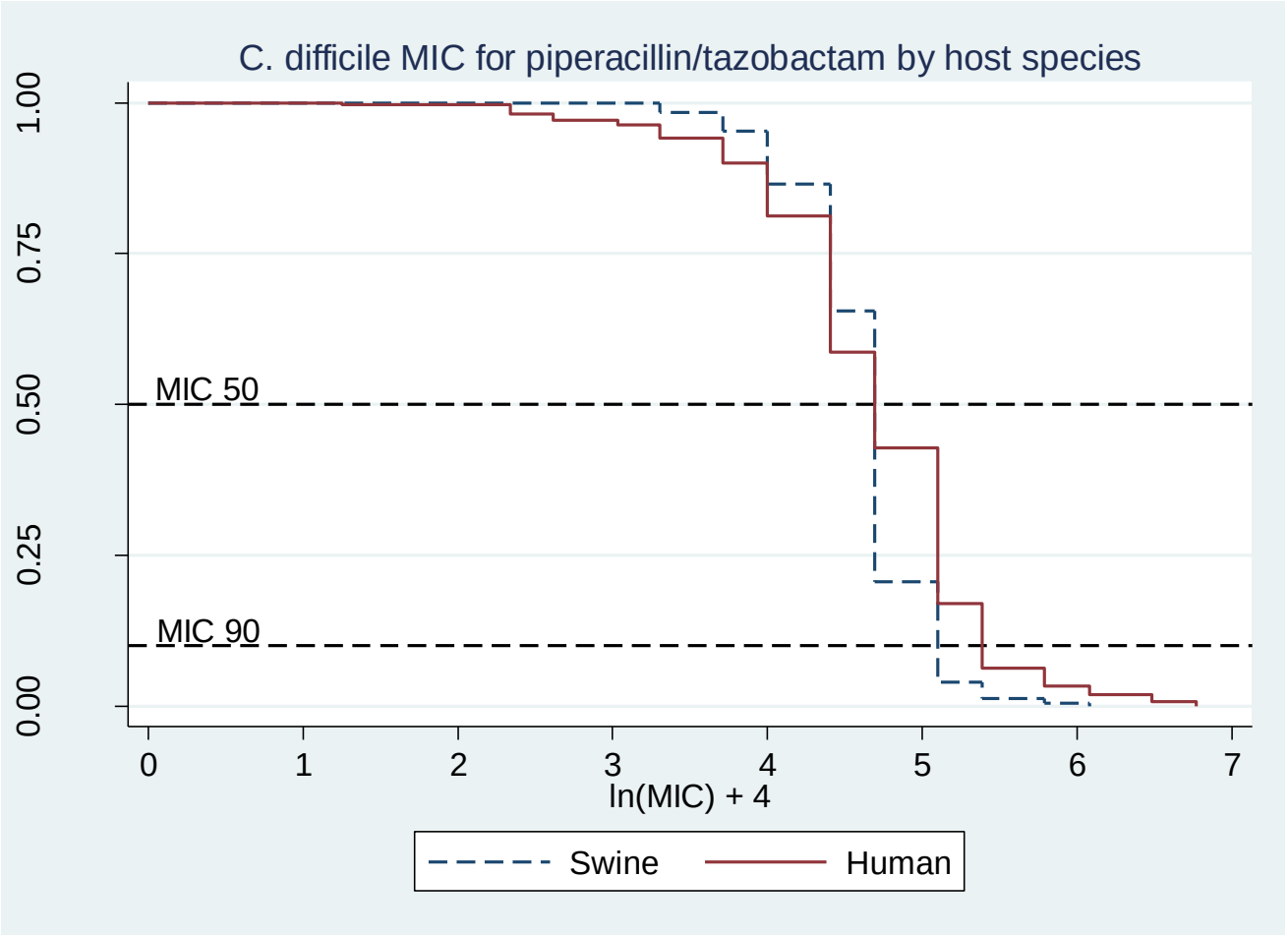

Figure 33. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for piperacillin/tazobactam by host species ($p<0.001$; log-rank test).

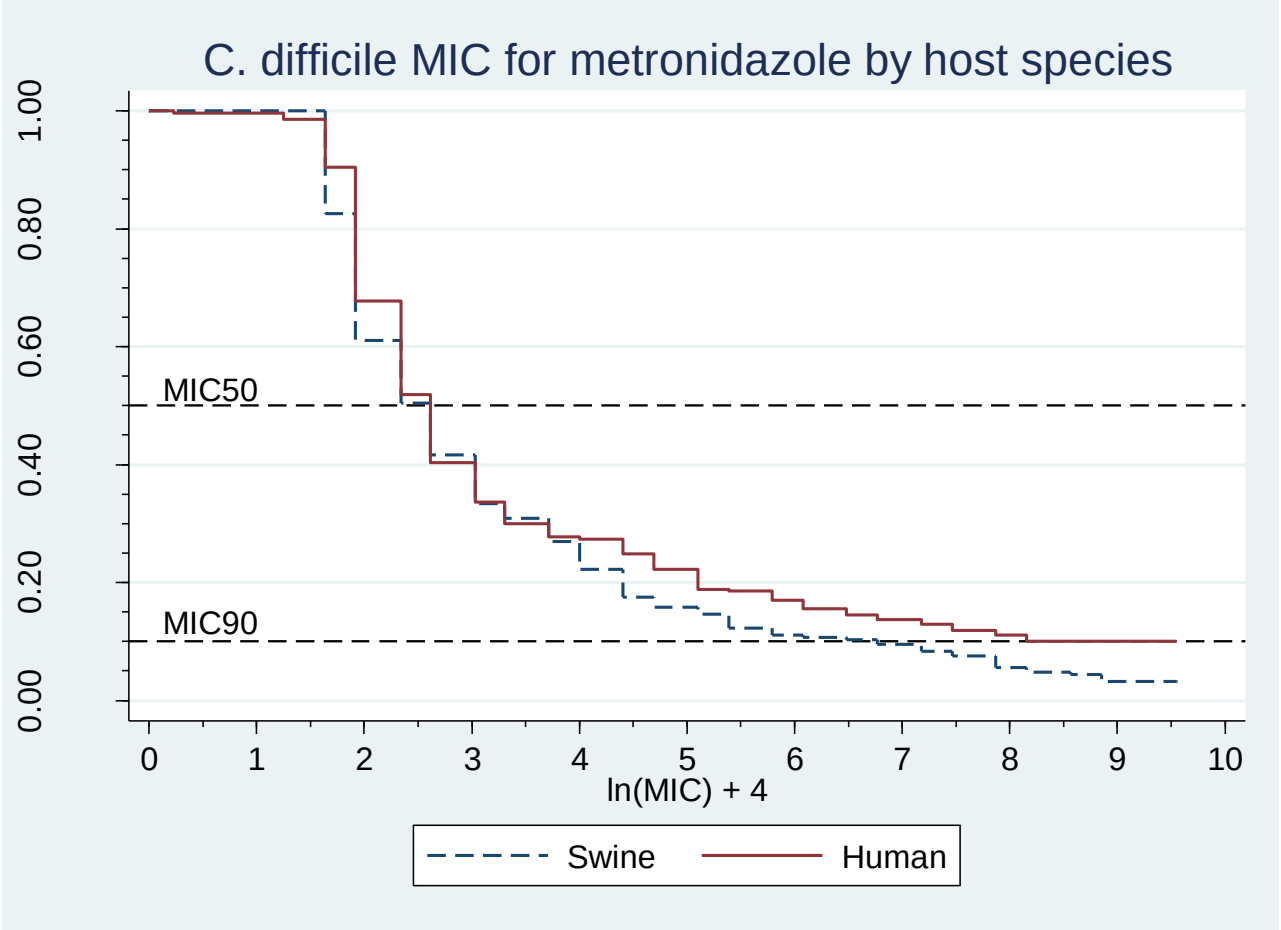

Figure 34. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for metronidazole by host species (p=0.03; log-rank test).

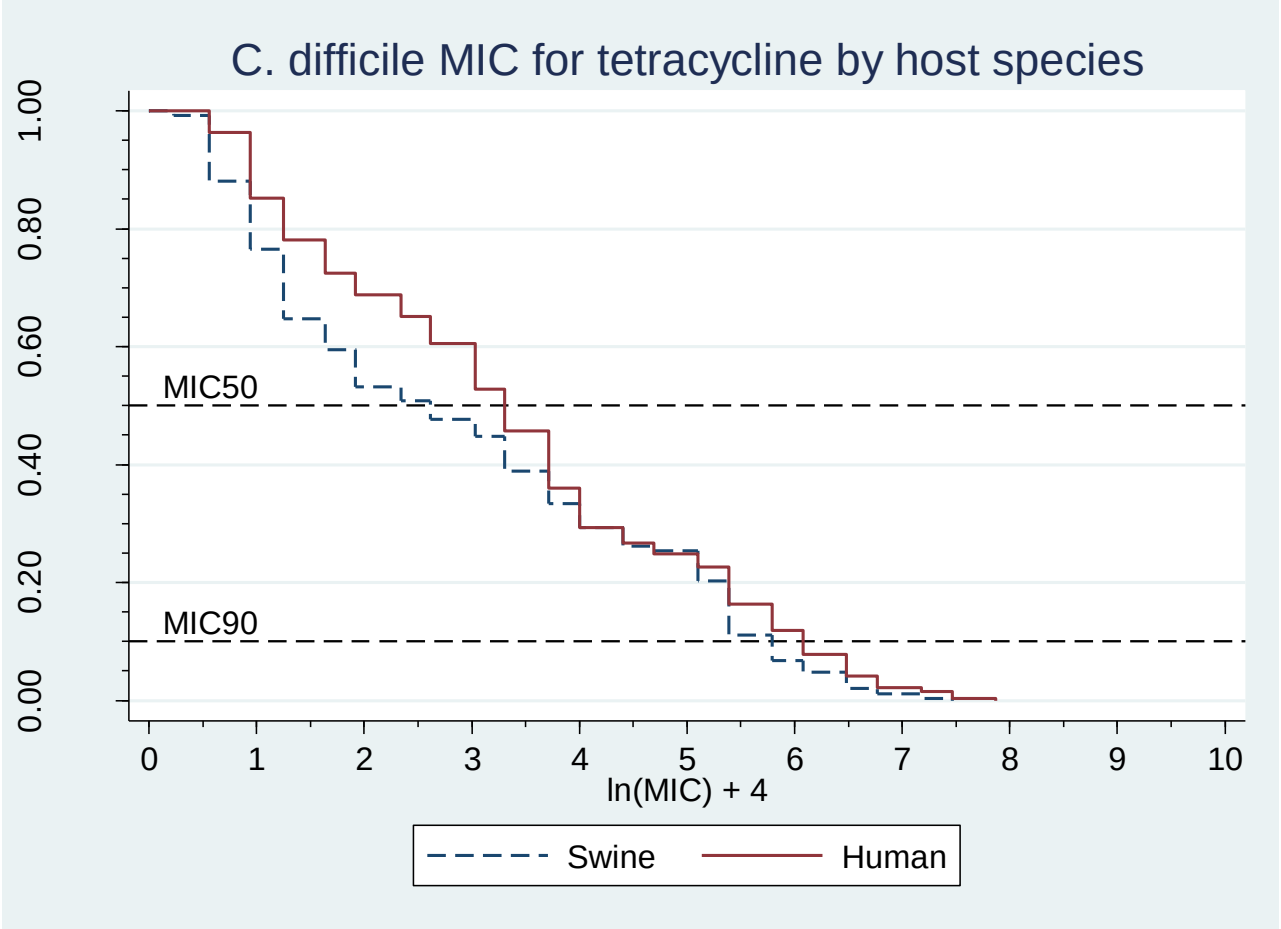

Figure 35. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for tetracycline by host species (p=0.02; log-rank test).

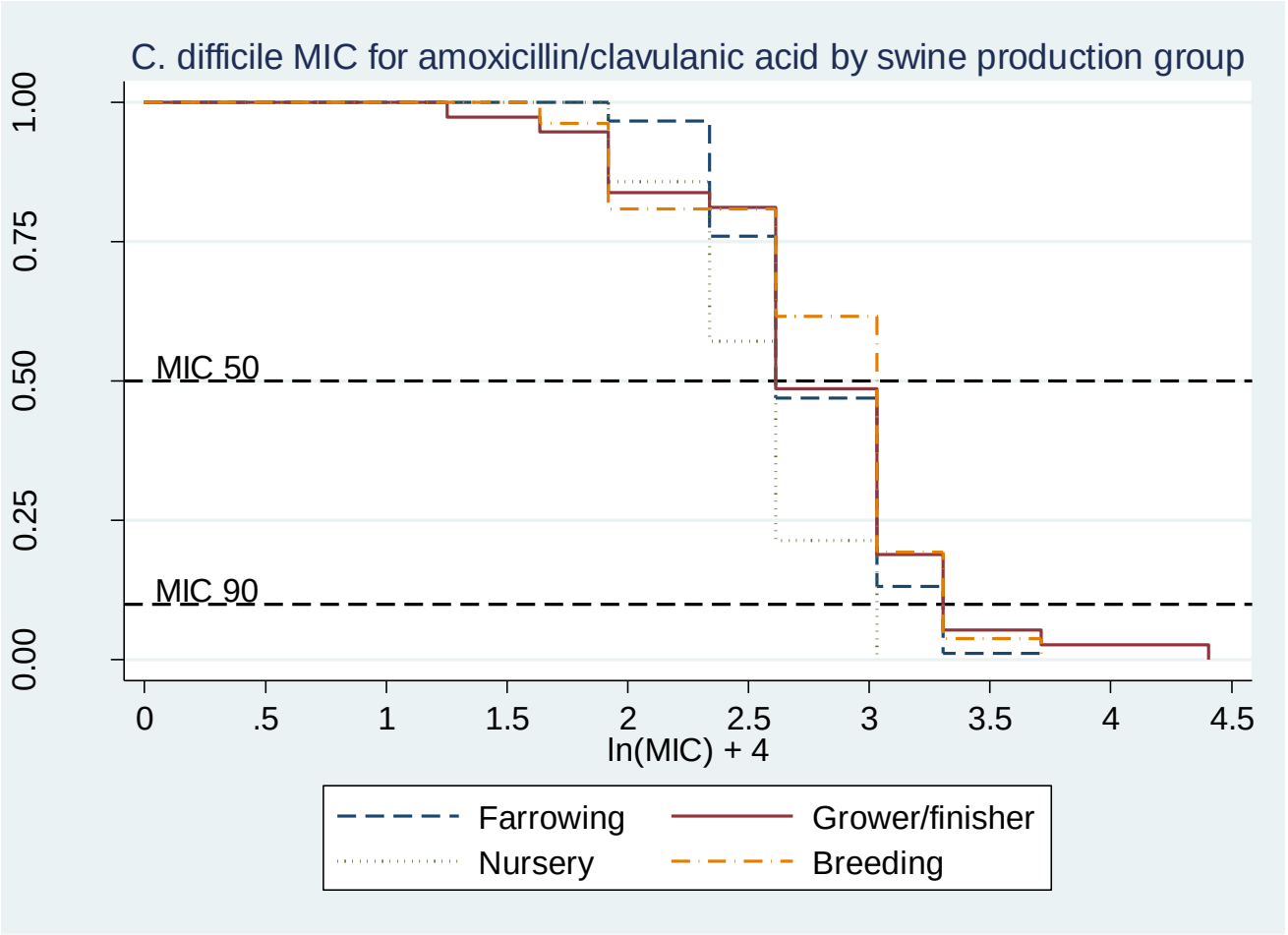

Figure 36. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for amoxicillin/clavulanic acid by swine production group (p=0.04; log-rank test).

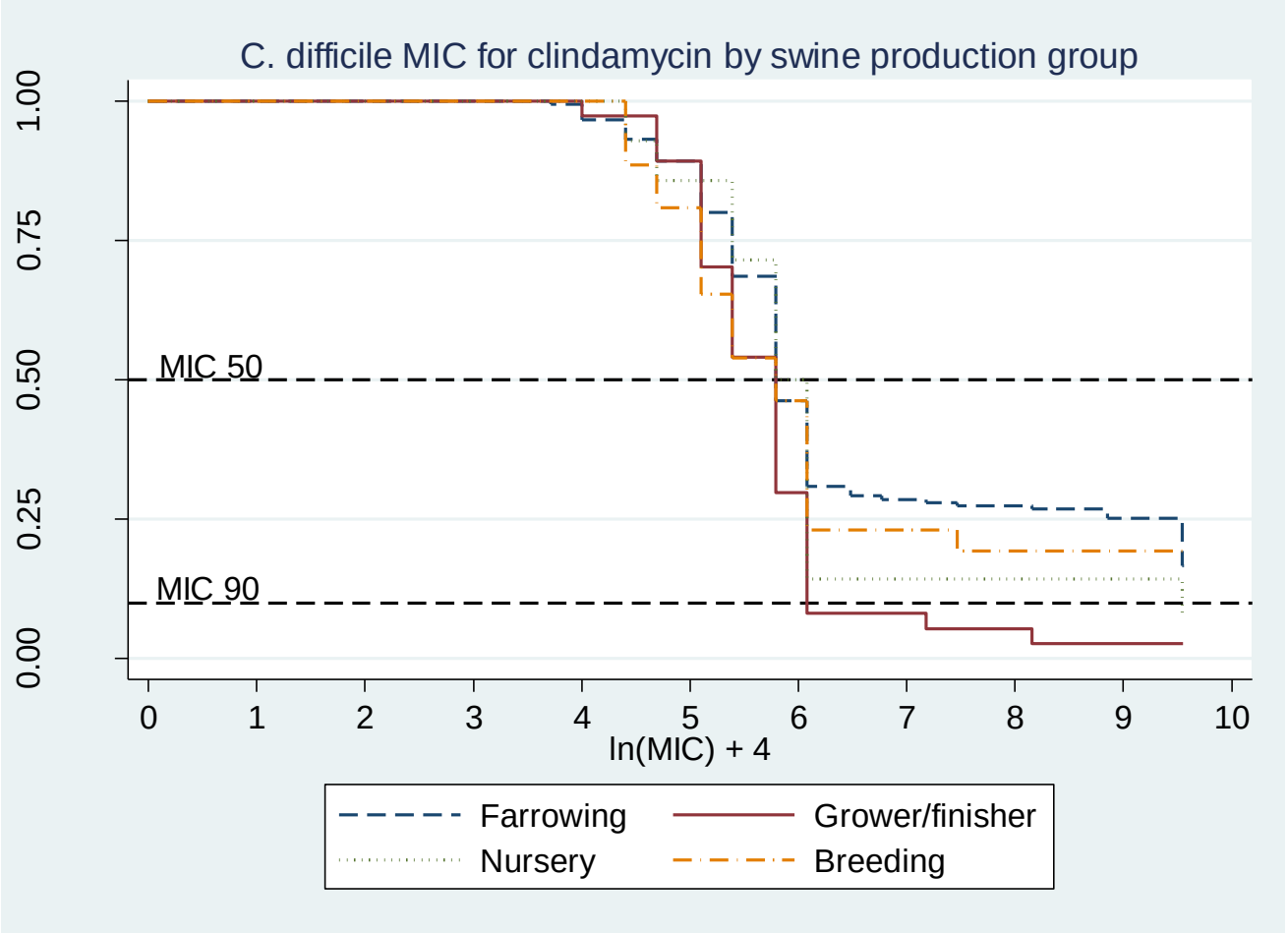

Figure 37. Non-parametric survival analysis graph of *Clostridium difficile* isolate MIC values for clindamycin by swine production group (p=0.04; log-rank test).

5. DISCUSSION

5.1 Discussion of results

The objectives of this study were to compare the prevalence and genotypic characteristics of *Clostridium difficile* from 2004 to 2006 in a closed and integrated human and swine population in Texas. The prevalence of *C. difficile* in the various swine production groups was compared to the prevalence in the human populations to explore the possibility of human infections occurring due to food-borne exposure; the prevalence and strain types in the swine worker cohort were compared to the non-worker cohort to explore possible occupational exposure; and isolates from the swine and human samples were compared to determine genotypic similarity of isolate carriage among the two host species overall.

The study population used to explore these hypotheses is unique, because it was a closed population, with little movement of host species (swine or human) in or out of the system, and contains an integrated population of both humans and swine. Previously published studies regarding transmission of *C. difficile* between food animals and humans have compared isolates from separate populations (54, 100-102, 122). It is difficult to make comparisons and inferences concerning transmission between species from separate populations, because each population has a different set of risk factors, and more importantly, potentially different distributions of confounders. Differences in strain carriage may simply be due to geography or demographics, and carriage of similar strains may be a result of a common point source rather than transmission between the

species; indeed, this latter hypothesis bears further exploration in all closely situated populations given the spore-forming ability of this organism. This is the first study to explore the potential transmission of *C. difficile* between food animals and humans in the same closed population of food animals and humans.

Previous studies evaluating the transmission of *C. difficile* from food animals to humans have focused on transmission via infected food animals (53, 54, 101, 102, 122, 123) or contamination of retail meat (48, 50, 56, 103, 123, 134-136). Studies of occupational risks of *C. difficile* have been limited to testing clinicians in health care facilities (49). Studies have investigated *C. difficile* strains found in food animals (54, 100-102, 122), therapeutic dogs (125, 126), and companion animals (dogs, horses, and cats) (100-102, 127, 128); however, no studies have investigated the risk of direct occupational exposure to food animals.

This is the first study to assess the occupational risk of *C. difficile* infection from food animals; specifically, from exposure to swine. The unique design of this study allowed the investigation of *C. difficile* in both a swine worker group cohort and swine non-worker group cohort. The swine worker and non-worker cohorts were housed separately, facilitating the collection of wastewater samples for each cohort and valid comparisons between the cohorts. Although the cohorts were housed separately, each matched pair was co-located within the same unit location, and experienced similar living conditions. Indeed, the food source was common to both groups. Importantly, there was little to no difference between the swine workers and non-workers other than their occupation.

Twelve of the human units sampled during the study contained a corresponding swine population; and the thirteenth human unit sampled was associated with the swine slaughter facility. All of the swine were born, raised, and slaughtered within the system, except for some purebred boars that entered the system for breeding purposes. These were held in quarantine for several months before being sent to each of the farrow-to-finish facilities.

The majority of published studies investigating *C. difficile* in swine have focused on piglets. *Clostridium difficile* is known to cause diarrhea, pseudomembranous colitis, and enteritis in piglets (87, 96-99); however, there are few data on the prevalence of *C. difficile* in older, slaughter-age swine. It is not known whether piglets with *C. difficile* continue to harbor the bacterium as they age or if, similar to human infants, the bacterium is replaced by other natural gut flora. The prevalence of *C. difficile* in grower/finisher pigs needed to be determined in order to more fully evaluate the potential risks due to food-borne exposure. The current study compared the prevalence of *C. difficile* in different production groups including farrowing barn inhabitants (sows and their suckling piglets), nursery (weaners), breeding (boars and dry sows), and grower/finisher swine. Ours is the first study to compare *C. difficile* among production groups and especially to determine the prevalence of *C. difficile* among asymptomatic grower/finisher swine.

This was a longitudinal study with repeated measurements of the outcome and exposure variables for samples repeatedly arising from the same group cohorts over the 3-year study period. This differs from a traditional cohort study, because of the

unknown status of the outcome of interest among the study subjects at the start of the study, and the cohort was not simply followed until some change in disease status occurred. If study subjects are known to not harbor the outcome of interest at the start of the investigation then the outcome measurement is incidence. In the current study, the status of *C. difficile* in the human and swine populations was not known at the beginning, nor could it be reliably inferred between sample periods, so the outcome at each time point is instead a repeated measurement of point prevalence. One of the advantages of cross-sectional studies is that multiple hypotheses regarding 'risk' factors or associations can be investigated. In the current study, the prevalence of *C. difficile* was observed in the different swine production groups and human occupational group cohorts as well as any seasonal trends in *C. difficile* prevalence. Cross-sectional studies are also well-suited for time-invariant exposure measures such as the swine production group and human occupational group cohort, and the random effect of unit location. The two major limitations of a cross-sectional study are that prevalence is the only measureable outcome and it is difficult to determine if the exposure (risk factor) of interest actually occurred before the outcome was measured (for time-variant exposures only). Due to these limitations it is very difficult to prove causation using a cross-sectional study (144). The current study was not designed to prove causation; instead, it was intended to assess the prevalence of *C. difficile* among swine production groups and human occupational group cohorts and determine if associations existed that would warrant concern for occupational or food-borne exposure to the bacterium. Finding trends in *C. difficile* carriage among the swine production groups or human occupational

group cohorts would necessitate further individual-level follow-up studies into the direct measure of transmission of *C. difficile* from swine to humans through food-borne or occupational exposure.

The samples in the current study were collected and analyzed at the group level rather than the individual level. These aggregated samples were collected at the pen level in the swine population and at the occupational group cohort level for the human population (by unit). Swine samples were classified as farrowing, nursery, breeding, or grower/finisher based on the barn and pen from which they were collected. Human wastewater samples were classified as swine worker or swine non-worker based on the location of the manhole from which they were collected. Studies in which samples are collected and the results are analyzed at the group level are classified as ecologic studies. There are several reasons for conducting ecologic studies including: measurement constraints at the individual level, homogeneity of the exposure, interest in group-level effects, and simplicity of analysis (144). Studying *C. difficile* at the group level was preferred in this study because of several of these factors: 1) there were ethical and legal problems with sampling humans at the individual level, 2) the relative variability within the swine production groups and human occupational group cohorts was expected to be small and, therefore, the exposure (group cohorts) would be fairly homogenous, 3) food-borne or occupational *C. difficile* infections were likely to be population-based problems, especially in our integrated population, and 4) based on preliminary data, the prevalence of *C. difficile* in non-farrowing production groups was likely to be low and therefore analyzing at a group level would help increase the statistical power in the analysis (145).

Ecological fallacy occurs when inferences are made at the individual level using data that have been collected and analyzed at the group level (144). Ecological fallacy is not a problem in the current study because the objective was to compare the prevalence between production group cohorts in the swine population and the occupational group cohorts in the human population, without inferring association at the individual-animal or human level. However, care must be taken in the presentation of the results and understanding that from these data the effects for individual swine or humans cannot be inferred. This results, from among many issues, the fact that a single positive result arising from a composite sample could be the result of one positive pig/human, several, or even all of them.

The highest prevalence of *C. difficile* was found in samples from the farrowing barn (Table 6). Samples from the farrowing barns were made up primarily (>95%) of feces from suckling piglets, but also may have contained traces of lactating sow feces. The swine veterinarian tasked with collecting these samples was instructed to collect piglet feces; however, we cannot be absolutely certain that there was never any contamination from the lactating sow in each of the farrowing crates. These results are consistent with other studies that have found a high prevalence of *C. difficile* among piglets. Two studies conducted in Iowa each detected *C. difficile* in the colon of 29% (97) and 42% (117) of piglets with symptoms of diarrhea, respectively. *C. difficile* was detected in 24.9% of the farrowing barn composite fecal samples in the current study. The prevalence reported from the current study is slightly lower than those found in the studies in Iowa; however, this may be entirely attributable to the different types of

samples used. Fecal samples were used in the current study whereas the authors of the other studies used samples from the colon obtained during necropsy of clinically ill piglets. Both of the studies mentioned above sampled piglets with symptoms of diarrhea and in the current study asymptomatic piglets were sampled. Interestingly, one of the studies conducted in Iowa also found *C. difficile* in the colon of 79.3% of asymptomatic piglets (117). The prevalence of *C. difficile* found in the colon of asymptomatic piglets in their study was once again much higher than the 24.9% prevalence we found in the feces, and this difference remains unexplained.

Alvarez-Perez et al. (2009) isolated *C. difficile* from 28.6% of the rectal swabs taken from asymptomatic piglets (1 to 7 days old) and 22.8% of the rectal swabs taken from symptomatic piglets (118). Their study is more comparable to the current study because the authors generated their prevalence estimates from rectal swabs of asymptomatic piglets. The 28.6% prevalence they found is very close to the 24.9% found in the current study. The study by Alvarez-Perez also evaluated the prevalence of *C. difficile* in both symptomatic and asymptomatic piglets that were 1 to 2 months old. They did not detect *C. difficile* in any of the rectal swabs from the 1 to 2 month old piglets. This is also consistent with the results in the current study that showed the prevalence of *C. difficile* declined significantly in older swine. In the current study, the prevalence declined from 24.9% in the farrowing barn to 5.1% in the nursery, 4.3% in the breeding barn, and 2.7% in the grower/finisher swine (Table 6). The 1 to 2 month old piglets mentioned in Alvarez-Perez et al. (2009) would be similar to the nursery production group in the current study (118). A total of 276 fecal samples were collected

from the nurseries in the current study and Alvarez-Perez et al. (2009) collected 239 rectal swab samples (118). Although the sample size numbers are comparable, the samples in the current study are aggregated and therefore account for more piglets overall. This may be one of the reasons a higher prevalence was found among the nursery piglets; however, it clearly is not the only reason. Increasing the number of piglets sampled may increase the probability of detecting a lower prevalence of *C. difficile*; however this is not necessarily the case. Another reason for detection of *C. difficile* in the nursery piglets in the current study is the culturing techniques used to isolate the bacterium. Alvarez et al. (2009) used an alcohol-shock treatment followed by plating on selective media (118). In the current study an alcohol-shock treatment was used, followed by a 7 day enrichment procedure, a second alcohol-shock treatment, and then plating to selective media. The enrichment procedure used in the current study should increase the probability of detection (i.e., increases the test sensitivity of the culture method). The study conducted by Alvarez et al. (2009) included both asymptomatic and symptomatic piglets; whereas the current study only included asymptomatic animals (118). We would expect the inclusion of symptomatic piglets in the Alavarez et al. study to increase the detection probability for *C. difficile*; however, another study in calves isolated *C. difficile* from 14.9% of asymptomatic and 7.6% of symptomatic calves (95); hence, assumptions about the relationship between clinical illness and probability of recovery of *C. difficile* may not hold true in all circumstances.

During an initial pilot study a significantly ($p<0.05$) higher prevalence of *C. difficile* was found arising from swine composite fecal samples during the fall and winter

months (September, October, November; December, January, February) (145). The estimated prevalence was 16.2% during the fall and winter months and 10.3% during the spring and summer months (March through August). That pilot study included 1,008 swine fecal samples from 2006 only. When the additional swine samples were included from 2004 and 2005 a significant difference in the prevalence of *C. difficile* was not found during either the winter months ($p=0.65$; compared to summer) or among the four separate seasons ($p=0.96$) when adjusting for the random effects of unit and year (Table 29). There have been no other published studies suggesting that there are seasonal trends in the prevalence of *C. difficile* in swine; however, one study reported a significantly ($p<0.05$) increased prevalence of *C. difficile* in retail meat sampled during the months of January and February when compared to March through August (50). Finding an increased prevalence of *C. difficile* in retail meat during the winter months would suggest that similar trends should be found among food animals if they are, in fact, the source of the meat contamination. A small sample size and disproportionate monthly sampling could be responsible for the differences in monthly prevalence in the aforementioned retail meat study (46) and further studies are needed to confirm or refute any reported seasonal trends of *C. difficile* in retail meat, as well as in feces. Seasonal trends in enteric pathogen-carriage among food animals are not uncommonly reported; however, most studies have found an increase in prevalence during the warmer months. As very limited and brief illustrative examples, one study reported that cattle shed *Campylobacter* spp. more heavily in the spring and autumn (146) while another found an increased prevalence of *E. coli* O157:H7 in cattle feces during the summer months (147).

Clostridium difficile has not previously been studied in human wastewater samples. An overall *C. difficile* prevalence of 11.8% was estimated in all of the human wastewater samples (Table 10). A study on the levels of human pathogens in biosolids from wastewater facilities detected *C. difficile* in 25 to 38% of their sludge samples (106). Biosolids are domestic sewer sludge that have been treated for pathogen removal and are then used for agricultural purposes. It is not known whether the high prevalence of *C. difficile* found in the biosolids was due to removal of other competing pathogens or whether biosolids provided a favorable environment for *C. difficile*; however, the latter hypothesis may be more plausible, perhaps due to the bacterium's spore-forming capacity. It is difficult to compare the findings from the current study on human wastewater samples (almost entirely water with little organic matter) to those arising from biosolids; however, both studies found a surprisingly high prevalence of *C. difficile* in human-sourced fecal matter. It has been estimated that 3% of healthy adults are asymptomatic carriers of *C. difficile* (2). The human population sampled in the current study was comprised of asymptomatic individuals and the individual-level prevalence in human wastewater samples (were that achievable) would be expected to be closer to the prevalence in asymptomatic adults. However since the samples in the current study were aggregated samples, rather than individual samples, this may help explain the higher prevalence. Another reason that the prevalence in the wastewater samples may have been higher than expected is that the contribution of each individual's feces to the whole is unknown, as well as the subsequent dynamics of bacteria growth in the contents of a wastewater sample. Wastewater samples may contain components that can either

enhance or hinder the survival of *C. difficile*. Alcohol-shock treatment has been shown to enhance the recovery of *C. difficile* spores (133). Wastewater samples may contain alcohol or other cleaning agents that promote the sporulation of *C. difficile*. The wastewater samples may also contain bleach, which will kill vegetative *C. difficile* and its spores and therefore may be a reason for the lowered prevalence found in the wastewater samples as compared to the biosolids.

Studies on asymptomatic carriers of *C. difficile* have tended to focus on patients in hospitals and nursing homes. One study found that 47.9% of the individuals sampled in a long-term care facility were asymptomatic carriers of *C. difficile* (105). Another study found that 11.9% of the infants (< 12 months old) and 21.1% of the children (1-11 yrs. old) sampled at a hospital in Thailand were asymptomatic carriers (104). It is difficult to compare the results from the current study with the results from the previously mentioned studies because their study subjects were in a hospital where there is an increased risk of acquiring *C. difficile*. The samples were also collected from individuals, compared to the aggregate samples used in the current study. Also, fecal samples were used to test for asymptomatic carriers in the hospital studies and fecal samples likely have a different diagnostic sensitivity than wastewater samples.

A significantly ($p<0.05$) increased prevalence of *C. difficile* in the wastewater samples was found during the spring, which included the months of March, April, and May (Table 12). There have been conflicting results regarding seasonal trends of *C. difficile* in humans while in hospitals. A study conducted in Denmark found no seasonal difference in the carriage of *C. difficile* in hospitalized children (81), similarly another

study found no seasonal trends in a teaching hospital in Australia (148). In contrast, a study conducted at the Veteran Administration Puget Sound Health Care System (VAPSHCS) in Seattle, Washington found that *C. difficile* followed a biannual seasonal pattern with an increased incidence both in the fall and spring (149). The results from the current study are somewhat similar to the results found at the VAPSHCS in that the highest prevalence of 16.3% was found in the spring; however, a higher prevalence in the fall was not found. The prevalence in the fall, winter, and summer months were all approximately 10.0% (Table 12). The difference in seasonal trend results may be due to the study population or even the geography (i.e, the Pacific Northwest versus southeast Texas). The study populations for the Denmark, Australia, and VAPSHCS studies were all hospitalized patients whereas our study population is likely more representative of the general population-at-large in the community. Finally, all 4 of the studies were conducted in different geographical regions and unmeasured but varying conditions may be entirely responsible for the observed seasonal pattern differences in *C. difficile* carriage.

No significant ($p=0.42$) difference was found in the prevalence of *C. difficile* in wastewater samples arising from swine worker and swine non-worker housing (Table 11). This is the first study to assess the risk of occupational exposure to swine and *C. difficile* infection. It has been shown that there is an increased risk of occupational exposure to *C. difficile* for health care workers in the human clinical setting (124). *C. difficile* was isolated from 23% of health care workers in wards that contained patients infected with *C. difficile,* and 6% of workers in wards without patients infected with *C.*

difficile. Although occupational exposure to *C. difficile* appears to be a concern in clinical settings, evidence that occupational exposure to swine is a risk factor for *C. difficile* in human wastewater samples was not found in the current study. A reason that occupational exposure to *C. difficile* may have been found in the clinical setting is that study did not adjust for the statistical dependence of results for staff assigned within ward (124). In the current study, multi-level mixed logistic regression was used to adjust for statistical dependence of wastewater samples by unit. In the clinical study, there may have been other differences among the wards that caused the increase in *C. difficile* prevalence, other than the presence of patients infected with *C. difficile*; adjustment for these differences were not included in the analysis. A second reason a difference in prevalence between workers and non-workers may not have been observed is that the farms may adhere to biosecurity measures that limit the exposure of workers to harmful pathogens. Another reason a difference may not have been observed is that human wastewater samples from representative manholes were utilized to compare the worker and non-worker populations. *C. difficile* prevalence in human wastewater samples is likely not representative of the prevalence within the individuals comprising the study population. It is more likely that the prevalence from aggregated samples is higher than individual prevalences, because the wastewater samples from non-carriers may be contaminated with *C. difficile* by wastewater samples from carriers. As discussed previously, it is also not known whether wastewater samples contain items that may hinder or promote the detection of *C. difficile*.

At the farm level, the highest prevalence of *C. difficile* in swine was found in the farrow-to-finish units (Table 9). The prevalence among the farrow-to-finish units (1, 6, 7, 5, and 3) was 14.6%, 14.5%, 11.6%, 10.9%, and 1.9%, respectively. It is not known why Unit 3 had a much lower prevalence than the other 4 farrow-to-finish units. Some of the reasons for the lower prevalence may be different management practices, differences in antibiotic use, or (perhaps, though unlikely) geographical location. *C. difficile* is an environmental contaminant and spores from infected swine could easily be spread by workers and equipment in the barns. Biosecurity measures to restrict contamination of pathogens such as *E. coli* and *Salmonella* in the units may also be effective for controlling the spread of *C. difficile*. No studies have been conducted to assess the risk of antibiotic use in swine and *C. difficile* infection; however, antibiotic use is the largest risk factor for infection in humans. Swine and humans share similar anatomical and physiological features; therefore, antibiotic use may plausibly be explored as an important risk factor for *C. difficile* infection in swine. The current study did not pursue this hypothesis; however, future studies should consider assessing antibiotic use, even if aggregated at the group level over longer periods such as a month or season.

There were no apparent differences in the prevalence of *C. difficile* in the human wastewater samples when comparing units (Table 14) as might be expected due to obvious differences such as swine farm type. The highest prevalence in humans (17.2%) was observed in Unit 7 (grower-finisher unit). Interestingly, Unit 3, which had the lowest prevalence among the farrow-to-finish units for swine, had the second highest

prevalence for the human wastewater samples. It was also found that units with a high prevalence of *C. difficile* in swine did not necessarily have a high prevalence in humans (Table 15). A difference in prevalence between swine workers and non-workers was not found and this could explain why a significant correlation between swine and human prevalence was not observed when comparing units.

The most prevalent toxinotype among the swine isolates was toxinotype V. Toxinotype V isolates are characterized by a 39-bp deletion in the *tcdC* gene, are positive for the binary toxin gene, and are positive for both the toxin A and toxin B genes. A total of 93.7% of the isolates were toxinotype V and there was no significant difference in toxinotype prevalence in the swine isolates between units ($p=0.18$) or season ($p=0.07$). However, there was a significant ($p<0.05$) difference in toxinotype prevalence between swine production groups (Figure 5). Toxinotype V was found in all 4 of the production groups; however, other toxinotypes were found in only 2 or 3 of the production groups. The finding of toxinotype V as the dominant toxinotype in the swine isolates is consistent with other reports (53, 122). A study conducted in the Netherlands observed that all *C. difficile* isolates sampled from the swine farms were toxinotype V (122), while a study in Slovenia estimated 76.7% (102/133) of swine isolates to be toxinotype V (53). The study in Slovenia, limited in scope and scale, sampled only three different farms and found that toxinotype V was found exclusively on two of the farms; whereas toxinotype 0 was found exclusively on another of the farms. In the current study, evidence of a difference in toxinotype carriage among units was not apparent. Since this is the first study to assess *C. difficile* across swine production

groups the findings of toxinotype differences between production groups cannot be compared with other studies. One of the reasons for the difference in toxinotypes may be due to the culturing techniques used in the current study. The culturing technique may favor the isolation of a specific toxinotype because it may have an unknown fitness advantage over the other toxinotypes during the enrichment procedure. There was also a large variation in the number of *C. difficile* isolates between production groups. The highest number of *C. difficile* isolates was found in the farrowing production group and this is the only production group to exhibit all 4 toxinotypes that were found in this study. At the other end of the spectrum, the nursery production group had the fewest number of *C. difficile* isolates and all of the isolates were found to be toxinotype V. Another reason a difference in toxinotypes may have been seen across production types is because of the small number of toxinotypes other than toxinotype V found in the study. The 3 toxinotypes other than toxinotype V only comprised 6.3% of the isolates.

Overall, 6 different PFGE patterns were identified among the swine isolates. The two most common PFGE patterns were NAP7 and a variant pattern that varied from NAP7 by only one band. The variant pattern was 90.5% similar to the NAP7 pattern (Figure 6). This pattern was actually more prevalent than NAP7, accounting for 68.7% of the isolates (Table 19). The NAP7 pattern accounted for 26.2% of the isolates. The remaining 4 PFGE patterns accounted for only 5.2% of the isolates. Combining type taxonomies (PFGE and toxinotype), the majority of our isolates belonged to the toxinotype V, PFGE NAP7-variant pattern, or else the toxinotype V, PFGE NAP7 pattern combination. These results are consistent with other studies that have observed

that the majority of *C. difficle* isolates from swine are of toxinotype V and PFGE type NAP7 (personal communication Angela Thompson, CDC). One study comparing toxinotype V isolates from humans and food animals reported that 6 of 14 swine isolates were NAP7 (54). The remaining isolates were NAP8 and were more than 70% similar to the NAP7 isolates. As in the study of toxinotype V isolates from humans and food animals (54), the current study found a high degree of similarity between the swine isolates. All of the isolates were more than 60% similar (Figure 7). A significant ($p<0.05$) difference in PFGE patterns was found across units and production groups when all the unknown PFGE patterns were collapsed into a single category (Figure 9 and 10). There are no published data concerning differences in PFGE patterns between farms or production groups; however, a previously mentioned study reported a difference in toxinotypes across three farms in Slovenia (53). Another study in Slovenia also found the majority of their swine isolates to be toxinotype V, ribotype 066 (53), whereas studies in the United States have found that the majority of swine isolates are toxinotype V, ribotype 078 (personal communication, Angela Thompson, CDC). These studies provide evidence that there may be very large geographical differences in strain carriage. Ribotyping analysis was not performed in our study so we do not know which ribotypes are among the swine isolates. The majority of the isolates are likely to be ribotype 078 based on a previous swine study conducted in the United States (101). Although a significant difference in strain carriage was found between swine units, this was largely due to the small number of strains that were not NAP7 (or its variant) and also the high degree of variability in detection across units. The remaining four

unknown PFGE patterns accounted for only 5.2% of the isolates. Even when all the unknown PFGE patterns were collapsed into a single category, there were PFGE patterns that were not found in many of the units. In addition, there was a high degree of variability in the number of isolates found in each unit. Only 1 or 2 isolates were detected in some of the units, whereas other units had close to 60 isolates each. A far greater number of isolates were isolated from the farrow-to-finish units compared to the grower-finisher units. Most of the difference in PFGE patterns across units can best be explained by the differences in the production groups.

The dominant toxinotype found among the human wastewater samples was also toxinotype V (Table 24). Although there was a greater diversity of toxinotypes among the human samples, 84.5% were toxinotype V. In addition to the toxinotypes found among the swine isolates, toxinotype III and three other unknown toxinotypes were found in the human samples. Toxinotype III strains are responsible for the recent severe outbreaks in hospitals in North America and Europe (33). Toxinotype V is not a strain that has been recognized as a major cause of disease in U.S. hospitals; however, it has been isolated from humans (150) and some studies have suggested that the rate of toxinotype V isolation is increasing (54). A significant difference in toxinotypes was not observed across season, unit, or occupational group cohort. In a study in the Netherlands, 14% of isolates from patients with CDAD were toxinotype V, 64% were toxinotype 0, and 16% were toxinotype III (150). This is very different than results of the current study which found that 84.5%, if isolates from wastewater draining housing representing healthy asymptomatic humans, were toxinotype V and 0.4% were

toxinotype III. No toxinotype 0 isolates were found; however, one of the three unknown toxinotypes may have been toxinotype 0. One of the reasons a higher prevalence of toxinotype V isolates may have been found is because the samples came from asymptomatic individuals and the samples from the Netherlands came from patients with CDAD. It has been suggested that certain strains of *C. difficile* may be responsible for community-acquired infection (130) and these may be the strains are found more commonly among the general public in the community. A study conducted in the Netherlands found that ribotype 078 (toxinotype V) was more often associated with community-acquired cases than ribotype 027 (toxinotype III) (130) and this finding could have important implications for understanding our data.

Among the human wastewater isolates, 17 different PFGE patterns were found; however, the majority of the isolates were NAP7 and the aforementioned NAP7-like variant pattern (Table 25). The variant pattern accounted for 66.8% of the isolates and 23.6% were NAP7. The remaining 15 unknown patterns only accounted for 26 (10.0%) of the isolates. There was a high degree of pulse-type similarity among the human wastewater isolates. There were two major phylogenic clusters that were more than 80% similar and all of the isolates were more than 75% similar. There was no significant difference in prevalence of PFGE pattern types among seasons, units, or between occupational group cohorts. It is difficult to compare the results from the current study to other studies is because most other studies have compared isolates from clinical human cases in health care facilities. Studies in human health care facilities have found PFGE type NAP1 (toxinotype III) to be the virulent strain responsible for recent

outbreaks in North America and Europe (151). While no studies of human clinical studies have made explicit mention of NAP7, studies have found ribotype 078, toxinotype V isolates among human cases and this is the strain most commonly associated with PFGE pattern NAP7 (130). Thus, the lack of reporting of NAP7 may simply reflect differences in typing preference, which does vary greatly around the world. As with toxinotype V isolates, it is unusual to find reports of a high prevalence of PFGE NAP7 isolates in human isolates; however, this may simply be due to the fact that the isolates from the current study are from asymptomatic individuals.

There were 29 unique combinations of PFGE pattern and toxinotype among the human wastewater samples (Table 26). Although there was a large diversity of strains, the majority of the strains were PFGE variant pattern and toxinotype V or PFGE NAP7 and toxinotype V combination. The PFGE NAP7-like variant pattern and toxinotype V combination accounted for 61.6% of the isolates and a further 22.1% were the NAP7 and toxinotype V. The remaining 27 combinations accounted for less than 2% of the isolates in total. It is common in studies of human clinical cases to find limited diversity of strains with one or two predominant strains; however, most often the dominant strain in human cases is not NAP7/toxinotype V (54, 101, 115, 130). A study conducted in the United States comparing animal and human isolates found that 12 different ribotypes among the human clinical cases and ribotype 078 (NAP7/toxinotype V) isolates accounted for only 4.4% of the isolates (101). Another study found that the most common ribotypes among human cases were 027 (NAP1/toxinotype III), ribotype 014, ribotype 078 (NAP7/toxinotype V), and ribotype 001; further, only 9% of the isolates

were ribotype 078 (NAP7/toxinotype V) (130). Finally, a study investigating toxinotype V isolates in the United States found 7 toxinotype V isolates among 6,000 isolates in VA hospitals from 1984 to 2001 and another 8 isolates out of 620 from hospital outbreaks from 2001 to 2007 (54). All of these studies provide evidence that while NAP7/toxinotype V strains have been found in humans; they are not at the high prevalence found in the current study. Contrary to previously mentioned studies, a study researching CA-CDAD found NAP1/toxinotype III isolates were the most prominent strain and the second most prominent strain was NAP7/toxinotype V (115). This latter study is likely more comparable to the results of the current study because the samples were from community-acquired cases rather than hospital-aquired cases. We propose that one of the reasons the current study may have found a high prevalence of NAP7/toxinotype V strains is because this strain is more commonly associated with CA-CDAD and asymptomatic carriers.

A high degree of similarity was found between swine and human strains. The majority of both the swine and human isolates were the variant or NAP7 PFGE pattern and toxinotype V (Table 27). All of the isolates in both populations were more than 70% similar. When comparing a dendrogram of the swine isolates (Figure 7) to a dendrogram with both host species (Figure 15), it was found that the swine isolates with an unknown PFGE pattern were more similar to human isolates than they were to other unknown PFGE swine isolates. However, there was a significant difference in the presence of binary toxin gene, *tcdC* gene deletion size, and toxinotype among the isolates across host species. The reason for the differences between host species is likely

because of the greater diversity of strains among the human isolates. All of the swine isolates were positive for the binary toxin, whereas only 90% of the human isolates were positive for the binary toxin. Isolates with a 39-bp deletion and no deletion in the *tcdC* gene were found among the swine isolates. These two deletions were found in the human isolates as well, along with an 18-bp deletion. A total of 4 toxinotypes were found among the swine isolates and 7 toxinotypes were found among the human isolates. Three of the toxinotypes found in the swine isolates were also found in the human isolates. This is not the first study to find similarities between human and swine strains of *C. difficile*. A study conducted on human clinical cases from VA hospitals and recent hospital outbreaks found toxinotype V human isolates that were 100% similar to swine isolates (54). Another study conducted in the Netherlands compared human clinical isolates to those from asymptomatic and symptomatic piglets. They compared 11 piglet and 21 human strains using MLVA and found that all of the strains were genetically related. The researchers also found that two pairs of human and swine isolates were 100% homologous (122, 129). The biggest difference between our study and the previously mentioned studies is that our swine and human populations were both contained within the same closed system and in close geographical proximity. The previously published studies in the United States and the Netherlands were each comparing human and swine strains that arose from completely different study populations and this makes it difficult to interpret any association between *C. difficile* infection in humans and food animal sources (54, 122, 129). Similar strain carriage between host species in the same study population provides some evidence for possible

transmission between species; equally plausible, the evidence suggests that a common point source may exist for both host species within the same geographical region.

Although similar strain carriage was found between the two host species in the present study, evidence of a low risk of infection via occupational and foodborne exposures was found. One reason for this may be that infection can occur from a common environmental source. *C. difficile* spores can survive in the environment for long periods of time under adverse conditions. *C. difficile* may be a ubiquitous environmental contaminant, and the more places we look for it, the more places we will find it. Until recently, *C. difficile* research has largely been confined to clinical settings. As research continues outside these clinical settings we continue to find *C. difficile* in new places. It is unknown whether C. *difficile* has always occurred in these places or if evolution of the bacterium has led to its expansion outside of the clinical setting.

Another reason for apparently similar strain carriage between the two host species it that our isolation methods may select for certain strains, making them appear similar even if the ratio of strains within hosts difers. There are no standardized protocols for the isolation of *C. difficile* and a variety of different methods have been previously published for isolation from animal fecal samples (95) and retail meat(48, 56). Methods differ by the media used, enrichment times, and alcohol or heat shock treatments. It is also not known if wastewater samples select for specific strains of *C. difficile*. Even if using wastewater to sample the human population selects for specific strains, this would likely not affect the degree of similarity found between the occupational group cohorts. Isolation of *C. difficile* from animal feces has been well

documented; however this is the first study to assess *C. difficile* from human wastewater samples. There is no reason to believe wastewater samples select for specific strains of *C. difficile* given the higher degree of variability found in the strains from the wastewater samples than from the swine samples.

Multilevel mixed-effects logistic regression models are essential tools in performing data analysis where aggregation of samples occurs. Mixed-effects models allow independent variables to be classified as either fixed or random effects. Random effect variables may have an effect on the dependent variable; however, the nature of this effect is often considered a nuisance rather than an estimated factor. Data were collected from 12 swine units and one additional human unit. There are most likely unmeasured differences that exist between these units; for example, differing management techniques in the swine units. It is unknown how such differences in the units may affect the prevalence of *C. difficile*; however, it is necessary to take into account the potential for clustering of (or, correlation among) responses at the unit level. Thus, a random-intercept model suffices for these purposes to account for dependence among responses and adjust for unknown or unmeasured unit-level effects. Prevalence data varied significantly between years for both host species; therefore, in the multilevel mixed-effects model, year was also included as a random effect. Including unit and year as random effects in the models accounted for the dependency of responses both by location and over time.

Multivariable and multilevel mixed-effects logistic regression models for the swine population illustrated that production group added significantly ($p<0.001$) to the

model when unit and year were included as random effects (Table 29). However, season and month were not found to be significant ($p>0.05$). A large component of the variance in *C. difficile* prevalence that was attributed to the unit in an intercept-only model could later be explained by the production group differences. In the intercept only model, 54.4% of the variance was attributed to the unit, while in the final model that included the production groups only 32.3% of the variance was attributed to the unit. The units with the highest prevalence of *C. difficile* were the farrow-to-finish units; on the other hand, the grower-finisher unit samples typically had much lower prevalence. As previously mentioned, the high prevalence of *C. difficile* in piglets has been well documented. The finding that there is a significant difference in production groups in models predicting *C. difficile* prevalence provides potential evidence of a lowered food safety risk in older slaughter-aged animals.

In the human population, multivariable and multilevel mixed-effects logistic regression models suggested that season was significantly associated with prevalence ($p=0.002$),when unit and year were included as random effects (Table 31). Occupational group cohort and month were unimportant. No studies have been published concerning the occupational risk of *C. difficile* infection and exposure to swine in the workplace. The finding that occupational group cohort was not significant in models estimating *C. difficile* prevalence provides evidence that occupational exposure is not likely to be a risk factor for *C. difficile* infection.

Multilevel mixed-effects logistic regression models across host species found that the fixed effects of host species and swine production group/human occupational

group cohort were significantly associated with *C. difficile* prevalence ($p<0.05$). Note, however, that the major factor differences were between host species rather than within host species, especially as regards human samples. Season did not contribute significantly to the model; however, the interaction of host species and season were important contributions to the model, hence season was also included in the model. Season was important in the human population but not significant ($p<0.05$) for the swine population; therefore, it was important to include season and the interaction of season and host species in the two-host model (Table 32). Since swine production group/human occupational group cohort is a more exhaustive version of the host species variable, swine production group/human occupational group cohort were included in a separate model with season (Table 33). Somewhat surprisingly, higher overall *C. difficile* prevalence was found in the human population, rather than the swine population. Some speculative reasons for this may be the type of samples collected or the methods used to isolate the bacterium. Both the swine fecal samples and the human wastewater samples were aggregated samples that represent a group of individuals. The big difference in the sample collection is that isolation of *C. difficile* from swine fecal samples has been well documented (118, 123, 152), whereas the current study is the first to broadly quantify *C. difficile* isolated from upstream human wastewater sources.

The nature of any chemicals or other compounds present in wastewater samples that may have hindered or promoted the growth, preservation, or detection of *C. difficile* is not known. There was also a difference in the plating step of the isolation methods used for the swine and wastewater samples. For the swine samples, 600 µl of

sterile de-ionized water was added to the enriched sediment before plating; for the human wastewater samples, only 200 µl of sterile de-ionized water was added to the sediment. Less water was added to the wastewater samples because a small pellet of sediment resulted from the sample, whereas the swine samples resulted in a larger pellet that needed to be diluted with more water in order to pipette the sample onto selective agar. The increased dilution to the swine samples could also be responsible for the lower prevalence resulting when compared with the human wastewater samples.

Analysis of the binary antimicrobial data found that all of the human and swine isolates were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, and vancomycin (Tables 34 and 36); in addition, all of the human isolates were susceptible to chloramphenical (Table 36). The majority of swine isolates was susceptible to ampicillin, chloramphenical, clindamyacin, metronidazole, and tetracycline (Table 34); whereas the majority had decreased susceptibility to cefoxitin, ciprofloxacin, and imipenem (Table 34). The majority of the human isolates were susceptible to ampicillin, clindamycin, imipenem, metronidazole (interpretation of results for metronidazole are under review) and tetracycline (Table 36); and the majority had decreased susceptibility to cefoxitin and ciprofloxacin (Table 36). There were significantly more swine isolates with decreased susceptibility to ampicillin, clindamycin, and imipenem and significantly ($p<0.05$) more human isolates with decreased susceptibility to ciprofloxacin when comparing between host species (Table 38).

Comparing swine production groups, a significant ($p<0.05$) difference in susceptibility was found for ciprofloxacin and tetracycline (Table 35). All swine isolates

arising from the nursery production group had decreased susceptibility to ciprofloxacin and tetracycline, while all isolates from the grower/finisher swine had decreased susceptibility to tetracycline. No significant difference was found between human occupational group cohorts. The antimicrobial usage data for the swine and human populations were not assessed in this study; however, varying rates of usage between and within the two populations may be responsible for the differences between and within host species (153). The finding of differences in susceptibility patterns between host species and the finding of no difference between human occupational group cohorts would provide evidence that transmission between host species is unlikely.

A study in the Netherlands comparing human and animal *C. difficile* strains found both similar as well as contrasting results (122). In contrast to the results of the current study, they found similar patterns for clindamycin between host species; similar to the results of the current study, they found patterns for metronidazole and vancomycin to be consistent between species. Another study conducted in the Netherlands also found no significant difference in antimicrobial resistance patterns between humans and swine (130). This latter study found that 94% of 49 isolates were resistant to ciprofloxacin and 57% were susceptible to clindamycin. The percentage of ciprofloxacin resistance reported in that study is similar to the 96% resistance we found among our human isolates. The majority of the isolates (77.6%) in the study from the Netherlands were human strains and this may be why the percent resistant to clindamycin more closely resembles our human results, rather than the 86.5% resistance we found in the swine isolates. However, this would not explain why the 57%

susceptible to clindamycin in the Dutch study more closely resembles the 56% susceptible in the swine isolates from the current study, rather than the 87.8% susceptible in the human isolates. Both of the studies conducted in the Netherlands had a much smaller sample size than the current study and this may be an additional reason why they did not find a statistically significant difference between host species. It is also important to note that the human and swine populations compared in the two studies from the Netherlands did not arise from the same integrated system.

Valuable data may be lost when collapsing antimicrobial data into binary categories. In addition to analyzing the binary data, survival analysis was used to assess the data across the actual MIC values. Similar to results from the binary analysis, a significantly ($p<0.05$) decreased susceptibility was found among swine to ampicillin, clindamycin, and imipenem and significantly ($p<0.05$) decreased susceptibility to ciprofloxacin in the human isolates when comparing between host species. In addition, through the MIC analysis, which includes coverage of the sub-breakpoint or cut-off values, significantly ($p<0.05$) increased levels of MIC were found in the human isolates to cefoxitin, amoxicillin/clavulanic acid, piperacillin/tazobactam, metronidazole, and tetracycline. Significant ($p<0.05$) MIC differences were also found among swine production groups for amoxicillin/clavulanic acid and clindamycin. As with the binary data analysis, no significant differences were observed across human occupational group cohorts. Using survival analysis to analyze the antimicrobial data resulted in similar results to analysis of the binary data; however, additional findings below the cutpoints were also noted that would have been missed if only the binary data was analyzed.

These latter findings are most important in assessing the emergence of resistance (i.e., beyond the established breakpoint) before it becomes widespread.

The most common risk factor for *C. difficile* is antibiotic use (154). This relationship with antibiotic use makes it difficult to both prevent and treat *C. difficile* infections. In the 1970's *C. difficile* first was linked to the use of clindamycin. Health care practitioners began to decrease the use of clindamycin and in the 1980's and 1990's cephalosporins were more heavily prescribed (35). Fluoroquinolone use began in the late 1980's, and in 2001 a study linked ciprofloxacin use to CDAD (155). The high level of ciprofloxacin resistance in the human isolates in our study is not surprising because the problem of fluoroquinolone resistance in health care facilities has been well documented (156-159). One of these studies assessed the antimicrobial resistance of *C. difficile* strains found in European hospitals and found a high level of resistance to a variety of fluoroquinolones, including ciprofloxacin (156). Clindamycin resistance in *C. difficile* is often associated with the virulent strain (NAP1/ribotype 027/toxinotype III) responsible for recent outbreaks (160, 161). The finding of low levels of resistance to clindamycin in the human samples in our study is encouraging and most likely relates to fact that we only found one toxinotype III isolate. The antibiotics most commonly prescribed to treat CDAD are metronidazole and vancomycin. Treatment of CDAD will be complicated by the discovery of strains resistant to these antibiotics. All of our human isolates were susceptible to vancomycin; on the other hand, 12.9% of our isolates were resistant to metronidazole. Resistance to metronidazole appears to be increasing; often, initial treatment with metronidazole is unsuccessful in treating CDAD and

subsequent vancomycin treatment is needed (162). Despite this, the majority of reported studies have found no resistance to either vancomycin or metronidazole (163, 164). It is important to note that at the time of publication the interpretation of the results for metronidazole and imipenem was under review. Many of the isolates classified as resistant to metronidazole or imipenem based on the CLSI breakpoints, showed vastly different results between 24 and 48 hours. Many of the isolates had MIC values that were classified as susceptible (based on CLSI breakpoints) at 24 hours, but at 48 hours there would be additional hazy growth on the plate, resulting in MIC values that would then be classified as resistant. Differing results for metronidazole or imipenem between 24 and 48 hours has not previously been reported in the *C. difficile* literature. A random sample of isolates classified as resistant to metronidazole, will be tested again using a new batch of Etest strips, to ensure the previous results are accurate. One of the reasons we may have found decreased susceptibility to metronidazole in the isolates of the current study may be due to the strains found in this population. The toxinotype V strains found in this study are not one of the strains typically isolated from patients in a clinical setting. One hypothesis may be that toxinotype V strains have a decreased susceptibility to metronidazole in comparison to strains found in the clinical setting.

5.2 Future Work

The isolates from the current study are currently undergoing ribotyping analysis and MLVA analysis through collaborative agreements with the Ohio State University and the University of Pittsburgh, respectively. This will allow the comparison of PFGE, ribotyping, and MLVA results. It will also allow for the comparison of results from the

current study to other studies that have used ribotyping, and not PFGE, to determine similarity of strains. In addition, investigation into the antimicrobial susceptibility results for metronidazole and imipenem will continue. Researchers are currently sequencing isolates resistant to metronidazole and it will be interesting to determine similarities or differences between those isolates and the isolates from the current study.

Further research is needed to investigate the possible sources of community-acquired *C. difficile* infection. The current study provides evidence that occupational and food-borne exposures are less likely sources of these community-acquired infections. Continuing to research *C. difficile* outside of the health care facility setting will help further the understanding of community-acquired infections and may lead to new hypotheses on possible sources of infection.

6. CONCLUSIONS

The objectives of this study were to assess the possible food-borne and occupational exposure risks of *C. difficile* in an integrated human and swine population. This is the first study to explore the risk of *C. difficile* infection in humans arising from occupational exposure to swine. It is also the first study to examine the varied prevalence of *C. difficile* across different swine production groups. There have been several studies that have explored the potential transmission of *C. difficile* from food animals to humans, often focusing on food animal products in the form of retail meats (e.g., ready-to-eat, frozen, and fresh meats). Many of these studies have focused on comparing strains between the host species, then drawing conclusions on the direction of flow for strains exhibiting shared carriage. Generally, the limitations of those studies are 1) the strains from the different host species have originated from separate study populations and 2) when the food animals are swine, the study population has focused only on piglets. The current study is different from previous studies in that samples were collected from a closed population that contained both a human and swine population. The human population consisted of a swine worker group cohort and a swine non-worker group cohort that were housed separately from one another on each of 13 units. The swine population flowed vertically from the farrowing barn to the grower/finisher slabs and all swine were slaughtered and then pork products were consumed entirely within the system.

The prevalence of *C. difficile* among the swine production groups was assessed in order to determine the potential for risk of human infection due to food-borne exposure. *C. difficile* is a known cause of diarrhea and psuedomembranous colitis in piglets; however, little is known about the bacteria in the other swine production groups. Consistent with other studies, the highest prevalence of *C. difficile* was found in the farrowing barn. The prevalence of *C. difficile* declined significantly in the other production groups with the lowest prevalence in the grower/finisher swine. The lowered prevalence of *C. difficile* in the grower/finisher swine may be indicative of a diminished risk of food-borne exposure than was previously assumed. No seasonal trends in *C. difficile* carriage were found in the swine population.

The risk of *C. difficile* infection from occupational exposure to swine was assessed by comparing the prevalence of *C. difficile* in human wastewater samples arising from each of the swine worker and non-worker cohorts. No significant difference was found in the prevalence of *C. difficile* between the occupational group cohorts; this should be indicative of a low risk of human *C. difficile* infection specifically arising from occupational exposure to swine. However, a significant seasonal difference in the carriage of *C. difficile* was found among the human population, with a higher prevalence of *C. difficile* during the spring (March, April, and May). Seasonal patterns in bacteria carriage are not unusual and there have been conflicting results about the seasonal trends of *C. difficile* in hospitals. While a seasonal difference in carriage was found in samples arising from the human population, no seasonal trends were found in samples arising from the swine population. Exposure to environmental sources of *C.*

difficile and differences in these environments among host species also may explain variations in seasonal carriage.

Although no evidence of transmission of *C. difficile* via occupational or food-borne exposure was found, very similar strain carriage was found between the two host species. Many studies have found similar *C. difficile* strains in human and swine populations; however, the isolates arising from each of those host species were from different study populations, or else not formally sampled from the same targeted population. The results from the current study suggest that rather than focusing on the 'flow' of *C. difficile* between hosts, identifying a common environmental point source for this spore-forming gram negative bacterium might be a more prudent approach. Indeed, it is quite possible that food (and feed) could be a quite suitable fomite or delivery mechanism for the spores to reach both humans and swine, respectively. However, since this does not fit the classic public health construct of a foodborne pathogen, perhaps the term 'food vectored' pathogen would be better suited to this situation.

The results from our swine population are consistent with other studies that have found that the majority of swine isolates are toxinotype V, PFGE NAP7 (or else a variant pattern that is 90.5% similar). The finding that the majority of our human isolates were also of this same strain is surprising. Toxinotype V, PFGE NAP7 strains have been found in humans; however, not nearly at as high prevalence as found in our study. One reason similar strains may have been found in both host species is the isolation method used to culture the bacteria. The isolation method we used to culture *C.*

difficile may select for certain strains. Another reason for the similar strain carriage is that our human isolates came from asymptomatic individuals. It has been suggested that the strains responsible for community-acquired *C. difficile* are different than those in hospital-acquired cases. If asymptomatic carriers are responsible for community-acquired infections then they will share similar strain carriage. A third reason for the similar strain carriage between the two host species is that infection can occur from a common environmental source. *C. difficile* spores can survive in the environment for long periods of time under adverse conditions. *C. difficile* may be a ubiquitous environmental contaminant, and the more places we look for it, the more places we will find it.

Further research is needed to investigate the possible sources of community-acquired *C. difficile* infection and the component causes needed to propagate the strains associated with CDAD. The current study provides evidence that occupational and food-borne exposures are less likely sources of these community-acquired bacteria. Continuing to research *C. difficile* outside of the health care facility setting will help further the understanding of community-acquired infections and may lead to new hypotheses on possible sources of infection (necessary causes), and their relative contributions to CA-CDAD when compared to the other component (if insufficient) cuases of disease.

REFERENCES

1. Silva J, Jr. *Clostridium difficile* nosocomial infections--still lethal and persistent. Infect Control Hosp Epidemiol. 1994;15(6):368-70.

2. Weir E, Flegel K. Protecting against *Clostridium difficile* illness. CMAJ. 2005;172(9):1178.

3. Fordtran JS. Colitis due to *Clostridium difficile* toxins: underdiagnosed, highly virulent, and nosocomial. Proc (Bayl Univ Med Cent). 2006;19(1):3-12.

4. Hall IC, O'Toole E. Intestinal flora in new-born infants with a description of a new pathogenic anaerobe, *Bacillus difficilis*. American Journal of Diseases of Children. 1935;49(2):390-402.

5. Jump RL, Pultz MJ, Donskey CJ. Vegetative *Clostridium difficile* survives in room air on moist surfaces and in gastric contents with reduced acidity: a potential mechanism to explain the association between proton pump inhibitors and *C. difficile*-associated diarrhea? Antimicrob Agents Chemother. 2007;51(8):2883-7.

6. Fedorko DP, Williams EC. Use of cycloserine-cefoxitin-fructose agar and L-proline-aminopeptidase (PRO Discs) in the rapid identification of *Clostridium difficile*. J Clin Microbiol. 1997;35(5):1258-9.

7. Levett PN. Effect of antibiotic concentration in a selective medium on the isolation of *Clostridium difficile* from faecal specimens. J Clin Pathol. 1985;38(2):233-4.

8. Dupuy B, Sonenshein AL. Regulated transcription of *Clostridium difficile* toxin genes. Mol Microbiol. 1998;27(1):107-20.

9. Hammond GA, Johnson JL. The toxigenic element of *Clostridium difficile* strain VPI 10463. Microb Pathog. 1995;19(4):203-13.

10. Hundsberger T, Braun V, Weidmann M, Leukel P, Sauerborn M, von Eichel-Streiber C. Transcription analysis of the genes tcdA-E of the pathogenicity locus of *Clostridium difficile*. Eur J Biochem. 1997;244(3):735-42.

11. Curry SR, Marsh JW, Muto CA, O'Leary MM, Pasculle AW, Harrison LH. tcdC genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. J Clin Microbiol. 2007;45(1):215-21.

12. Geric B, Rupnik M, Gerding DN, Grabnar M, Johnson S. Distribution of *Clostridium difficile* variant toxinotypes and strains with binary toxin genes among clinical isolates in an American hospital. J Med Microbiol. 2004;53(Pt 9):887-94.

13. Smith LD, King EO. Occurrence of *Clostridium difficile* in infections of man. J Bacteriol. 1962;84:65-7.

14. George RH, Symonds JM, Dimock F, Brown JD, Arabi Y, Shinagawa N, et al. Identification of *Clostridium difficile* as a cause of pseudomembranous colitis. Br Med J. 1978;1(6114):695.

15. Torpy JM, Lynm C, Glass RM. *Clostridium difficile* colitis. JAMA. 2009;301(9):988.

16. Sell TL, Schaberg DR, Fekety FR. Bacteriophage and bacteriocin typing scheme for *Clostridium difficile*. J Clin Microbiol. 1983;17(6):1148-52.

17. Hachler H, Wust J. Reexamination by bacteriophage typing of *Clostridium difficile* strains isolated during a nosocomial outbreak. J Clin Microbiol. 1984;20(3):604.

18. Hawkins CC, Buggy BP, Fekety R, Schaberg DR. Epidemiology of colitis induced by *Clostridium difficile* in hamsters: application of a bacteriophage and bacteriocin typing system. J Infect Dis. 1984;149(5):775-80.

19. Tabaqchali S, O'Farrell S, Holland D, Silman R. Method for the typing of *Clostridium difficile* based on polyacrylamide gel electrophoresis of [35S]methionine-labeled proteins. J Clin Microbiol. 1986;23(1):197-8.

20. Tabaqchali S, Holland D, O'Farrell S, Silman R. Typing scheme for *Clostridium difficile*: its application in clinical and epidemiological studies. Lancet. 1984;1(8383):935-8.

21. Wren B, Heard SR, Tabaqchali S. Association between production of toxins A and B and types of *Clostridium difficile*. J Clin Pathol. 1987;40(12):1397-401.

22. Wren BW, Tabaqchali S. Restriction endonuclease DNA analysis of *Clostridium difficile*. J Clin Microbiol. 1987;25(12):2402-4.

23. Gurtler V. Typing of *Clostridium difficile* strains by PCR-amplification of variable length 16S-23S rDNA spacer regions. J Gen Microbiol. 1993;139(12):3089-97.

24. Chachaty E, Saulnier P, Martin A, Mario N, Andremont A. Comparison of ribotyping, pulsed-field gel electrophoresis and random amplified polymorphic DNA for typing *Clostridium difficile* strains. FEMS Microbiol Lett. 1994;122(1-2):61-8.

25. Kato H, Kato N, Watanabe K, Ueno K, Ushijima H, Hashira S, et al. Application of typing by pulsed-field gel electrophoresis to the study of *Clostridium difficile* in a neonatal intensive care unit. J Clin Microbiol. 1994;32(9):2067-70.

26. Kristjansson M, Samore MH, Gerding DN, DeGirolami PC, Bettin KM, Karchmer AW, et al. Comparison of restriction endonuclease analysis, ribotyping, and pulsed-field gel electrophoresis for molecular differentiation of *Clostridium difficile* strains. J Clin Microbiol. 1994;32(8):1963-9.

27. Rupnik M, Avesani V, Janc M, von Eichel-Streiber C, Delmee M. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J Clin Microbiol. 1998;36(8):2240-7.

28. Indra A, Huhulescu S, Schneeweis M, Hasenberger P, Kernbichler S, Fiedler A, et al. Characterization of *Clostridium difficile* isolates using capillary gel electrophoresis-based PCR ribotyping. J Med Microbiol. 2008;57(Pt 11):1377-82.

29. Killgore G, Thompson A, Johnson S, Brazier J, Kuijper E, Pepin J, et al. Comparison of seven techniques for typing international epidemic strains of *Clostridium difficile*: restriction endonuclease analysis, pulsed-field gel electrophoresis, PCR-ribotyping, multilocus sequence typing, multilocus variable-number tandem-repeat analysis, amplified fragment length polymorphism, and surface layer protein A gene sequence typing. J Clin Microbiol. 2008;46(2):431-7.

30. Bidet P, Lalande V, Salauze B, Burghoffer B, Avesani V, Delmee M, et al. Comparison of PCR-ribotyping, arbitrarily primed PCR, and pulsed-field gel electrophoresis for typing *Clostridium difficile*. J Clin Microbiol. 2000;38(7):2484-7.

31. van den Berg RJ, Schaap I, Templeton KE, Klaassen CH, Kuijper EJ. Typing and subtyping of *Clostridium difficile* isolates by using multiple-locus variable-number tandem-repeat analysis. J Clin Microbiol. 2007;45(3):1024-8.

32. Marsh JW, O'Leary MM, Shutt KA, Sambol SP, Johnson S, Gerding DN, et al. Multilocus variable number tandem repeat analysis and multilocus sequence typing reveal genetic relationships among *Clostridium difficile* isolates

genotyped by restriction endonuclease analysis. J Clin Microbiol. 2010;48(2):412-8

33. Kuijper EJ, Coignard B, Tull P. Emergence of *Clostridium difficile*-associated disease in North America and Europe. Clin Microbiol Infect. 2006;12 Suppl 6:2-18.

34. Pituch H, Obuch-Woszczatynski P, Wultanska D, Meisel-Mikolajczyk F, Luczak M. A survey of metronidazole and vancomycin resistance in strains of *Clostridium difficile* isolated in Warsaw, Poland. Anaerobe. 2005;11(4):197-9.

35. Gerding DN. Clindamycin, cephalosporins, fluoroquinolones, and *Clostridium difficile*-associated diarrhea: this is an antimicrobial resistance problem. Clin Infect Dis. 2004;38(5):646-8.

36. McDonald LC, Killgore GE, Thompson A, Owens RC, Jr., Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med. 2005;353(23):2433-41.

37. Barbut F, Gariazzo B, Bonne L, Lalande V, Burghoffer B, Luiuz R, et al. Clinical features of *Clostridium difficile*-associated infections and molecular characterization of strains: results of a retrospective study, 2000-2004. Infect Control Hosp Epidemiol. 2007;28(2):131-9.

38. Drudy D GB, Bakker D, Kyne L, van den Berg R, Fenelon L, et al. . Clindamycin-resistant clone of *Clostridium difficile* PCR Ribotype 027, Europe. Emerging Infectious Disease. 2008;14(9):1485-7.

39. Dupuy B, Govind R, Antunes A, Matamouros S. *Clostridium difficile* toxin synthesis is negatively regulated by TcdC. J Med Microbiol. 2008;57(Pt 6):685-9.

40. Spigaglia P, Mastrantonio P. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J Clin Microbiol. 2002;40(9):3470-5.

41. Warny M, Pepin J, Fang A, Killgore G, Thompson A, Brazier J, et al. Toxin production by an emerging strain of *Clostridium difficile* associated with outbreaks of severe disease in North America and Europe. Lancet. 2005;366(9491):1079-84.

42. Rupnik M, Grabnar M, Geric B. Binary toxin producing *Clostridium difficile* strains. Anaerobe. 2003;9(6):289-94.

43. Geric B, Carman RJ, Rupnik M, Genheimer CW, Sambol SP, Lyerly DM, et al. Binary toxin-producing, large clostridial toxin-negative *Clostridium difficile* strains are enterotoxic but do not cause disease in hamsters. J Infect Dis. 2006;193(8):1143-50.

44. Barbut F, Decre D, Lalande V, Burghoffer B, Noussair L, Gigandon A, et al. Clinical features of *Clostridium difficile*-associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains. J Med Microbiol. 2005;54(Pt 2):181-5.

45. Huang H, Weintraub A, Fang H, Nord CE. Community acquired *Clostridium difficile* infection due to a moxifloxacin susceptible ribotype 027 strain. Scand J Infect Dis. 2009;41(2):158-9.

46. CDC. Surveillance for community-associated *Clostridium difficile*-Connecticut. MMWR. 2006;57(13):340-3.

47. Dalton BR, Lye-Maccannell T, Henderson EA, Maccannell DR, Louie TJ. Proton pump inhibitors increase significantly the risk of *Clostridium difficile* infection in a low-endemicity, non-outbreak hospital setting. Aliment Pharmacol Ther. 2009;29(6):626-34.

48. Songer JG, Trinh HT, Killgore GE, Thompson AD, McDonald LC, Limbago BM. *Clostridium difficile* in retail meat products, USA, 2007. Emerg Infect Dis. 2009;15(5):819-21.

49. Bouza E, Martin A, Van den Berg RJ, Kuijper EJ. Laboratory-acquired *Clostridium difficile* polymerase chain reaction ribotype 027: a new risk for laboratory workers? Clin Infect Dis. 2008;47(11):1493-4.

50. Rodriguez-Palacios A, Reid-Smith RJ, Staempfli HR, Daignault D, Janecko N, Avery BP, et al. Possible seasonality of *Clostridium difficile* in retail meat, Canada. Emerg Infect Dis. 2009;15(5):802-5.

51. Zidaric V, Zemljic M, Janezic S, Kocuvan A, Rupnik M. High diversity of *Clostridium difficile* genotypes isolated from a single poultry farm producing replacement laying hens. Anaerobe. 2008;14(6):325-7.

52. Simango C, Mwakurudza S. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int J Food Microbiol. 2008;124(3):268-70.

53. Pirs T, Ocepek M, Rupnik M. Isolation of *Clostridium difficile* from food animals in Slovenia. J Med Microbiol. 2008;57(Pt 6):790-2.

54. Jhung MA, Thompson AD, Killgore GE, Zukowski WE, Songer G, Warny M, et al. Toxinotype V *Clostridium difficile* in humans and food animals. Emerg Infect Dis. 2008;14(7):1039-45.

55. Rupnik M. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clin Microbiol Infect. 2007;13(5):457-9.

56. Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS. *Clostridium difficil*e in retail ground meat, Canada. Emerg Infect Dis. 2007;13(3):485-7.

57. al Saif N, Brazier JS. The distribution of *Clostridium difficile* in the environment of South Wales. J Med Microbiol. 1996;45(2):133-7.

58. Songer JG. The emergence of *Clostridium difficile* as a pathogen of food animals. Anim Health Res Rev. 2004;5(2):321-6.

59. Cunningham R, Dale B, Undy B, Gaunt N. Proton pump inhibitors as a risk factor for *Clostridium difficile* diarrhoea. J Hosp Infect. 2003;54(3):243-5.

60. Lefebvre SL, Reid-Smith R, Boerlin P, Weese JS. Evaluation of the risks of shedding *Salmonellae* and other potential pathogens by therapy dogs fed raw diets in Ontario and Alberta. Zoonoses Public Health. 2008;55(8-10):470-80.

61. Vonberg RP, Reichardt C, Behnke M, Schwab F, Zindler S, Gastmeier P. Costs of nosocomial *Clostridium difficile*-associated diarrhoea. J Hosp Infect. 2008;70(1):15-20.

62. Gravel D, Miller M, Simor A, Taylor G, Gardam M, McGeer A, et al. Health care-associated *Clostridium difficile* infection in adults admitted to acute care hospitals in Canada: a Canadian Nosocomial Infection Surveillance Program Study. Clin Infect Dis. 2009;48(5):568-76.

63. Raveh D, Rabinowitz B, Breuer GS, Rudensky B, Yinnon AM. Risk factors for *Clostridium difficile* toxin-positive nosocomial diarrhoea. Int J Antimicrob Agents. 2006;28(3):231-7.

64. Henoun Loukili N, Martinot M, Hansmann Y, Christmann D. [Risk factors for nosocomial *Clostridium difficile* diarrhoea in an infectious and tropical diseases department]. Med Mal Infect. 2004;34(2):57-61.

65. Safdar N, Maki DG. The commonality of risk factors for nosocomial colonization and infection with antimicrobial-resistant *Staphylococcus aureus*, *Enterococcus*, gram-negative bacilli, *Clostridium difficile*, and *Candida*. Ann Intern Med. 2002;136(11):834-44.

66. Barbut F, Petit JC. Epidemiology, risk factors and prevention of *Clostridium difficile* nosocomial infections. Pathol Biol (Paris). 2000;48(8):745-55.

67. Delmee M, Michaux JL, Williams M. Prevention of *Clostridium difficile* outbreaks in hospitals. Lancet. 1986;328(8502):350.

68. Ciesla WP, Bobak DA. Management and Prevention of *Clostridium difficile*-Associated Diarrhea. Curr Infect Dis Rep. 2001;3(2):109-15.

69. Ward CO. Diagnosis, treatment, and prevention of *Clostridium difficile* colitis. Consult Pharm. 2003;18(12):1050-4.

70. Jenkins L. The prevention of *Clostridium difficile* associated diarrhoea in hospital. Nurs Times. 2004;100(26):56-7, 9.

71. Katz JA. Probiotics for the prevention of antibiotic-associated diarrhea and *Clostridium difficile* diarrhea. J Clin Gastroenterol. 2006;40(3):249-55.

72. Pelleschi ME. *Clostridium difficile*-associated disease: diagnosis, prevention, treatment, and nursing care. Crit Care Nurse. 2008;28(1):27-35; quiz 6.

73. Tung JM, Dolovich LR, Lee CH. Prevention of *Clostridium difficile* infection with *Saccharomyces boulardii*: a systematic review. Can J Gastroenterol. 2009;23(12):817-21.

74. Gerding DN. Antimotility agents for the treatment of *Clostridium difficile* infection: is the juice worth the squeeze? Clin Infect Dis. 2009;48(5):606-8.

75. Hellemans R, Naegels S, Holvoet J. Fecal transplantation for recurrent *Clostridium difficile* colitis, an underused treatment modality. Acta Gastroenterol Belg. 2009;72(2):269-70.

76. Imhoff A, Karpa K. Is there a future for probiotics in preventing *Clostridium difficile*-associated disease and treatment of recurrent episodes? Nutr Clin Pract. 2009;24(1):15-32.

77. Leffler DA, Lamont JT. Treatment of *Clostridium difficile*-associated disease. Gastroenterology. 2009;136(6):1899-912.

78. Pareja Sierra T. *Clostridium difficile* infection in the elderly: update in epidemiology and treatment. Med Clin (Barc). 2009;133(5):180-3.

79. Rohde CL, Bartolini V, Jones N. The use of probiotics in the prevention and treatment of antibiotic-associated diarrhea with special interest in *Clostridium difficile*-associated diarrhea. Nutr Clin Pract. 2009;24(1):33-40.

80. Salazar M, Garey KW, Jiang ZD, Dao-Tran T, Dupont H. Changing *Clostridium difficile* infection testing and treatment trends at a large tertiary care teaching hospital. Pharm World Sci. 2009;31(5):565-71.

81. Tvede M, Schiotz PO, Krasilnikoff PA. Incidence of *Clostridium difficile* in hospitalized children. A prospective study. Acta Paediatr Scand. 1990;79(3):292-9.

82. Hirschhorn LR, Trnka Y, Onderdonk A, Lee ML, Platt R. Epidemiology of community-acquired *Clostridium difficile*-associated diarrhea. J Infect Dis. 1994;169(1):127-33.

83. Riley TV, Cooper M, Bell B, Golledge CL. Community-acquired *Clostridium difficile*-associated diarrhea. Clin Infect Dis. 1995;20 Suppl 2:S263-5.

84. Wilcox MH, Mooney L, Bendall R, Settle CD, Fawley WN. A case-control study of community-associated *Clostridium difficile* infection. J Antimicrob Chemother. 2008;62(2):388-96.

85. Bauer MP, Goorhuis A, Koster T, Numan-Ruberg SC, Hagen EC, Debast SB, et al. Community-onset *Clostridium difficile*-associated diarrhoea not associated with antibiotic usage--two case reports with review of the changing epidemiology of *Clostridium difficile*-associated diarrhoea. Neth J Med. 2008;66(5):207-11.

86. Dial S, Delaney JA, Barkun AN, Suissa S. Use of gastric acid-suppressive agents and the risk of community-acquired *Clostridium difficile*-associated disease. JAMA. 2005;294(23):2989-95.

87. Songer JG, Anderson MA. *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 2006;12(1):1-4.

88. Songer JG. Clostridia as agents of zoonotic disease. Vet Microbiol. 2010;140(3-4):399-404.

89. Aseeri M, Schroeder T, Kramer J, Zackula R. Gastric acid suppression by proton pump inhibitors as a risk factor for *Clostridium difficile*-associated diarrhea in hospitalized patients. Am J Gastroenterol. 2008;103(9):2308-13.

90. Dial S, Alrasadi K, Manoukian C, Huang A, Menzies D. Risk of *Clostridium difficile* diarrhea among hospital inpatients prescribed proton pump inhibitors: cohort and case-control studies. CMAJ. 2004;171(1):33-8.

91. Al-Tureihi FI, Hassoun A, Wolf-Klein G, Isenberg H. Albumin, length of stay, and proton pump inhibitors: key factors in *Clostridium difficile*-associated disease in nursing home patients. J Am Med Dir Assoc. 2005;6(2):105-8.

92. Muto CA, Pokrywka M, Shutt K, Mendelsohn AB, Nouri K, Posey K, et al. A large outbreak of *Clostridium difficile*-associated disease with an unexpected proportion of deaths and colectomies at a teaching hospital following increased fluoroquinolone use. Infect Control Hosp Epidemiol. 2005;26(3):273-80.

93. Pepin J, Saheb N, Coulombe MA, Alary ME, Corriveau MP, Authier S, et al. Emergence of fluoroquinolones as the predominant risk factor for *Clostridium difficile*-associated diarrhea: a cohort study during an epidemic in Quebec. Clin Infect Dis. 2005;41(9):1254-60.

94. Shah S, Lewis A, Leopold D, Dunstan F, Woodhouse K. Gastric acid suppression does not promote clostridial diarrhoea in the elderly. QJM. 2000;93(3):175-81.

95. Rodriguez-Palacios A, Stampfli HR, Duffield T, Peregrine AS, Trotz-Williams LA, Arroyo LG, et al. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg Infect Dis. 2006;12(11):1730-6.

96. Post KW, Songer JG. Antimicrobial susceptibility of *Clostridium difficile* isolated from neonatal pigs with enteritis. Anaerobe. 2004;10(1):47-50.

97. Yaeger M, Funk N, Hoffman L. A survey of agents associated with neonatal diarrhea in Iowa swine including *Clostridium difficile* and porcine reproductive and respiratory syndrome virus. J Vet Diagn Invest. 2002;14(4):281-7.

98. Waters EH, Orr JP, Clark EG, Schaufele CM. Typhlocolitis caused by *Clostridium difficile* in suckling piglets. J Vet Diagn Invest. 1998;10(1):104-8.

99. Jung K, Ha SK, Chung HK, Kim J, Cho WS, Choi C, et al. Archival PCR-based diagnosis of *Clostridium difficile* in piglets. Vet Rec. 2003;153(15):466-7.

100. Arroyo LG, Kruth SA, Willey BM, Staempfli HR, Low DE, Weese JS. PCR ribotyping of *Clostridium difficile* isolates originating from human and animal sources. J Med Microbiol. 2005 Feb;54(Pt 2):163-6.

101. Keel K, Brazier JS, Post KW, Weese S, Songer JG. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J Clin Microbiol. 2007;45(6):1963-4.

102. Bouvet PJ, Popoff MR. Genetic relatedness of *Clostridium difficile* isolates from various origins determined by triple-locus sequence analysis based on toxin regulatory genes tcdC, tcdR, and cdtR. J Clin Microbiol. 2008;46(11):3703-13.

103. Weese JS, Avery BP, Rousseau J, Reid-Smith RJ. Detection and enumeration of *Clostridium difficile* spores in retail beef and pork. Appl Environ Microbiol. 2009;75(15):5009-11.

104. Wongwanich S, Pongpech P, Dhiraputra C, Huttayananont S, Sawanpanyalert P. Characteristics of *Clostridium difficile* strains isolated from asymptomatic individuals and from diarrheal patients. Clin Microbiol Infect. 2001;7(8):438-41.

105. Riggs MM, Sethi AK, Zabarsky TF, Eckstein EC, Jump RL, Donskey CJ. Asymptomatic carriers are a potential source for transmission of epidemic and nonepidemic *Clostridium difficile* strains among long-term care facility residents. Clin Infect Dis. 2007;45(8):992-8.

106. Viau E, Peccia J. Survey of wastewater indicators and human pathogen genomes in biosolids produced by class a and class B stabilization treatments. Appl Environ Microbiol. 2009;75(1):164-74.

107. Rexach CE, Tang-Feldman YJ, Cantrell MC, Cohen SH. Epidemiologic surveillance of *Clostridium difficile* diarrhea in a freestanding pediatric hospital and a pediatric hospital at a university medical center. Diagn Microbiol Infect Dis. 2006;56(2):109-14.

108. Noren T, Akerlund T, Back E, Sjoberg L, Persson I, Alriksson I, et al. Molecular epidemiology of hospital-associated and community-acquired *Clostridium difficile* infection in a Swedish county. J Clin Microbiol. 2004;42(8):3635-43.

109. Terhes G, Urban E, Soki J, Hamid KA, Nagy E. Community-acquired *Clostridium difficile* diarrhea caused by binary toxin, toxin A, and toxin B gene-positive isolates in Hungary. J Clin Microbiol. 2004;42(9):4316-8.

110. Karlstrom O, Fryklund B, Tullus K, Burman LG. A prospective nationwide study of *Clostridium difficile*-associated diarrhea in Sweden. The Swedish C. difficile Study Group. Clin Infect Dis. 1998;26(1):141-5.

111. Levy DG, Stergachis A, McFarland LV, Van Vorst K, Graham DJ, Johnson ES, et al. Antibiotics and *Clostridium difficile* diarrhea in the ambulatory care setting. Clin Ther. 2000;22(1):91-102.

112. Pant C, Madonia P, Minocha A. Does PPI therapy predispose to *Clostridium difficile* infection? Nat Rev Gastroenterol Hepatol. 2009;6(9):555-7.

113. Delaney JA, Dial S, Barkun A, Suissa S. Antimicrobial drugs and community-acquired *Clostridium difficile*-associated disease, UK. Emerg Infect Dis. 2007;13(5):761-3.

114. Patel NS. Fluoroquinolone use is the predominant risk factor for the development of a new strain of *Clostridium difficile*-associated disease. BJU Int. 2007;99(6):1333-4.

115. Limbago BM, Long CM, Thompson AD, Killgore GE, Hannett GE, Havill NL, et al. *Clostridium difficile* strains from community-associated infections. J Clin Microbiol. 2009;47(9):3004-7.

116. Nagy J, Bilkei G. Neonatal piglet losses associated with *Escherichia coli* and *Clostridium difficile* infection in a Slovakian outdoor production unit. Vet J. 2003;166(1):98-100.

117. Yaeger MJ, Kinyon JM, Glenn Songer J. A prospective, case control study evaluating the association between *Clostridium difficile* toxins in the colon of neonatal swine and gross and microscopic lesions. J Vet Diagn Invest. 2007;19(1):52-9.

118. Alvarez-Perez S, Blanco JL, Bouza E, Alba P, Gibert X, Maldonado J, et al. Prevalence of *Clostridium difficile* in diarrhoeic and non-diarrhoeic piglets. Vet Microbiol. 2009;137(3-4):302-5.

119. Eglow R, Pothoulakis C, Itzkowitz S, Israel EJ, O'Keane CJ, Gong D, et al. Diminished *Clostridium difficile* toxin A sensitivity in newborn rabbit ileum is associated with decreased toxin A receptor. J Clin Invest. 1992;90(3):822-9.

120. Keel MK, Songer JG. The comparative pathology of *Clostridium difficile*-associated disease. Vet Pathol. 2006;43(3):225-40.

121. Kiss D, Bilkei G. A new periparturient disease in Eastern Europe, *Clostridium difficile* causes postparturient sow losses. Theriogenology. 2005;63(1):17-23.

122. Debast SB, van Leengoed LA, Goorhuis A, Harmanus C, Kuijper EJ, Bergwerff AA. *Clostridium difficile* PCR ribotype 078 toxinotype V found in diarrhoeal pigs identical to isolates from affected humans. Environ Microbiol. 2009;11(2):505-11.

123. Indra A, Lassnig H, Baliko N, Much P, Fiedler A, Huhulescu S, et al. *Clostridium difficile*: a new zoonotic agent? Wien Klin Wochenschr. 2009;121(3-4):91-5.

124. van Nood E, van Dijk K, Hegeman Z, Speelman P, Visser CE. Asymptomatic carriage of *Clostridium difficile* among HCWs: Do we disregard the doctor? Infect Control Hosp Epidemiol. 2009;30(9):924-5.

125. Lefebvre SL, Weese JS. Contamination of pet therapy dogs with MRSA and *Clostridium difficile*. J Hosp Infect. 2009;72(3):268-9.

126. Lefebvre SL, Arroyo LG, Weese JS. Epidemic *Clostridium difficile* strain in hospital visitation dog. Emerg Infect Dis. 2006;12(6):1036-7.

127. Riley TV, Adams JE, O'Neill GL, Bowman RA. Gastrointestinal carriage of *Clostridium difficile* in cats and dogs attending veterinary clinics. Epidemiol Infect. 1991;107(3):659-65.

128. Struble AL, Tang YJ, Kass PH, Gumerlock PH, Madewell BR, Silva J, Jr. Fecal shedding of *Clostridium difficile* in dogs: a period prevalence survey in a veterinary medical teaching hospital. J Vet Diagn Invest. 1994;6(3):342-7.

129. Goorhuis A, Debast SB, van Leengoed LA, Harmanus C, Notermans DW, Bergwerff AA, et al. *Clostridium difficile* PCR ribotype 078: an emerging strain in humans and in pigs? J Clin Microbiol. 2008;46(3):1157; author reply 8.

130. Goorhuis A, Bakker D, Corver J, Debast SB, Harmanus C, Notermans DW, et al. Emergence of *Clostridium difficile* infection due to a new hypervirulent strain, polymerase chain reaction ribotype 078. Clin Infect Dis. 2008;47(9):1162-70.

131. Weese JS, Rousseau J, Arroyo L. Bacteriological evaluation of commercial canine and feline raw diets. Can Vet J. 2005;46(6):513-6.

132. Broda DM, DeLacy KM, Bell RG, Braggins TJ, Cook RL. Psychrotrophic *Clostridium* spp. associated with 'blown pack' spoilage of chilled vacuum-packed red meats and dog rolls in gas-impermeable plastic casings. Int J Food Microbiol. 1996;29(2-3):335-52.

133. Marler LM, Siders JA, Wolters LC, Pettigrew Y, Skitt BL, Allen SD. Comparison of five cultural procedures for isolation of *Clostridium difficile* from stools. J Clin Microbiol. 1992;30(2):514-6.

134. Von Abercron SM, Karlsson F, Wigh GT, Wierup M, Krovacek K. Low occurrence of *Clostridium difficile* in retail ground meat in Sweden. J Food Prot. 2009;72(8):1732-4.

135. Weese JS, Reid-Smith RJ, Avery BP, Rousseau J. Detection and characterization of *Clostridium difficile* in retail chicken. Lett Appl Microbiol. 2010;50(4):362-5.

136. Jobstl M, Heuberger S, Indra A, Nepf R, Kofer J, Wagner M. *Clostridium difficile* in raw products of animal origin. Int J Food Microbiol. 2010;138(1):172-5.

137. Julien MC, Dion P, Lafreniere C, Antoun H, Drouin P. Sources of clostridia in raw milk on farms. Appl Environ Microbiol. 2008;74(20):6348-57.

138. Kyne L, Warny M, Qamar A, Kelly CP. Asymptomatic carriage of *Clostridium difficile* and serum levels of IgG antibody against toxin A. N Engl J Med. 2000;342(6):390-7.

139. Rivera EV, Woods S. Prevalence of asymptomatic *Clostridium difficile* colonization in a nursing home population: a cross-sectional study. J Gend Specif Med. 2003;6(2):27-30.

140. Bartlett JG. *Clostridium difficile*: history of its role as an enteric pathogen and the current state of knowledge about the organism. Clin Infect Dis. 1994;18 Suppl 4:S265-72.

141. Kato H, Kato N, Watanabe K, Iwai N, Nakamura H, Yamamoto T, et al. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J Clin Microbiol. 1998;36(8):2178-82.

142. Kato N, Ou CY, Kato H, Bartley SL, Brown VK, Dowell VR, Jr., et al. Identification of toxigenic *Clostridium difficile* by the polymerase chain reaction. J Clin Microbiol. 1991;29(1):33-7.

143. Rupnik M, Dupuy B, Fairweather NF, Gerding DN, Johnson S, Just I, et al. Revised nomenclature of *Clostridium difficile* toxins and associated genes. J Med Microbiol. 2005;54(Pt 2):113-7.

144. Dohoo I, Martin W, Stryhn H. Veterinary Epidemiologic Research Charlottetown, Prince Edward Island, Canada: AVC Inc.; 2003.

145. Norman KN, Harvey RB, Scott HM, Hume ME, Andrews K, Brawley AD. Varied prevalence of *Clostridium difficile* in an integrated swine operation. Anaerobe. 2009;15(6):256-60.

146. Stanley KN, Wallace JS, Currie JE, Diggle PJ, Jones K. The seasonal variation of thermophilic *Campylobacters* in beef cattle, dairy cattle and calves. J Appl Microbiol. 1998;85(3):472-80.

147. Edrington TS CT, Ives SE, Engler MJ, Looper ML, Anderson RC, et al. Seasonal shedding of *Escherichia coli* O157:H7 in ruminants: a new hypothesis. Foodborne Pathogens Dis 2006;3(4):413-21.

148. Riley TV, O'Neill GL, Bowman RA, Golledge CL. *Clostridium difficile*-associated diarrhoea: epidemiological data from Western Australia. Epidemiol Infect. 1994;113(1):13-20.

149. McFarland LV, Clarridge JE, Beneda HW, Raugi GJ. Fluoroquinolone use and risk factors for *Clostridium difficile*-associated disease within a Veterans Administration health care system. Clin Infect Dis. 2007;45(9):1141-51.

150. Paltansing S, van den Berg RJ, Guseinova RA, Visser CE, van der Vorm ER, Kuijper EJ. Characteristics and incidence of *Clostridium difficile*-associated disease in The Netherlands, 2005. Clin Microbiol Infect. 2007;13(11):1058-64.

151. O'Connor JR, Johnson S, Gerding DN. *Clostridium difficile* infection caused by the epidemic BI/NAP1/027 strain. Gastroenterology. 2009;136(6):1913-24.

152. Post KW, Jost BH, Songer JG. Evaluation of a test for *Clostridium difficile* toxins A and B for the diagnosis of neonatal swine enteritis. J Vet Diagn Invest. 2002;14(3):258-9.

153. Alali WQ, Scott HM, Christian KL, Fajt VR, Harvey RB, Lawhorn DB. Relationship between level of antibiotic use and resistance among *Escherichia coli* isolates from integrated multi-site cohorts of humans and swine. Prev Vet Med. 2009;90(3-4):160-7.

154. Fekety R, Kim KH, Batts DH, Browne RA, Cudmore MA, Silva J, Jr., et al. Studies on the epidemiology of antibiotic-associated *Clostridium difficile* colitis. Am J Clin Nutr. 1980;33(11 Suppl):2527-32.

155. Yip C, Loeb M, Salama S, Moss L, Olde J. Quinolone use as a risk factor for nosocomial *Clostridium difficile*-associated diarrhea. Infect Control Hosp Epidemiol. 2001;22(9):572-5.

156. Spigaglia P, Barbanti F, Mastrantonio P, Brazier JS, Barbut F, Delmee M, et al. Fluoroquinolone resistance in *Clostridium difficile* isolates from a prospective study of *C. difficile* infections in Europe. J Med Microbiol. 2008;57(Pt 6):784-9.

157. Drudy D, Harnedy N, Fanning S, Hannan M, Kyne L. Emergence and control of fluoroquinolone-resistant, toxin A-negative, toxin B-positive *Clostridium difficile*. Infect Control Hosp Epidemiol. 2007;28(8):932-40.

158. Adams DA, Riggs MM, Donskey CJ. Effect of fluoroquinolone treatment on growth of and toxin production by epidemic and nonepidemic *Clostridium difficile* strains in the cecal contents of mice. Antimicrob Agents Chemother. 2007;51(8):2674-8.

159. Drudy D, Quinn T, O'Mahony R, Kyne L, O'Gaora P, Fanning S. High-level resistance to moxifloxacin and gatifloxacin associated with a novel mutation in gyrB in toxin-A-negative, toxin-B-positive *Clostridium difficile*. J Antimicrob Chemother. 2006;58(6):1264-7.

160. Fenner L, Widmer AF, Stranden A, Conzelmann M, Goorhuis A, Harmanus C, et al. First cluster of clindamycin-resistant *Clostridium difficile* PCR ribotype 027 in Switzerland. Clin Microbiol Infect. 2008;14(5):514-5.

161. Kuijper EJ, Barbut F, Brazier JS, Kleinkauf N, Eckmanns T, Lambert ML, et al. Update of *Clostridium difficile* infection due to PCR ribotype 027 in Europe, 2008. Euro Surveill. 2008;13(31):1-7.

162. Huang H, Weintraub A, Fang H, Nord CE. Antimicrobial resistance in *Clostridium difficile*. Int J Antimicrob Agents. 2009;34(6):516-22.

163. Huang H, Fang H, Weintraub A, Nord CE. Distinct ribotypes and rates of antimicrobial drug resistance in *Clostridium difficile* from Shanghai and Stockholm. Clin Microbiol Infect. 2009;15(12):1170-3.

164. Taori SK, Hall V, Poxton I. The influence of antibiotics on the changing epidemiology of *Clostridium difficile*. J Med Microbiol. 2010;59:338.

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