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**Comparison of antimicrobial susceptibility among *Clostridium difficile* isolated from
an integrated human and swine population in Texas**

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Abstract

Clostridium difficile can be a major problem in hospitals because the bacterium primarily affects individuals with an altered intestinal flora; this largely occurs through prolonged antibiotic use. Proposed sources of increased community-acquired infections are food animals and retail meats. The objective of this study was to compare the antimicrobial resistance patterns of *C. difficile* isolated from a closed, integrated population of humans and swine to increase understanding of the bacterium in these populations. Swine fecal samples were collected from a vertically flowing swine population consisting of farrowing, nursery, breeding, and grower/finisher production groups. Human wastewater samples were collected from swine worker and non-worker occupational group cohorts. Antimicrobial susceptibility testing was performed on 523 *C. difficile* strains from the population using commercially available agar diffusion Epsilometer test (Etest®) for 11 different antimicrobials. All of the swine and human strains were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, and vancomycin. In addition, all the human strains were susceptible to chloramphenicol. The majority of the human and swine strains were resistant to cefoxitin and ciprofloxacin. Statistically significant differences in antimicrobial susceptibility were found among the swine production groups for ciprofloxacin, tetracycline, amoxicillin/clavulanic acid, and clindamycin. No significant differences in antimicrobial susceptibility were found across human occupational group cohorts. We found that 8.3% of the swine strains and 13.3% of the human strains exhibited resistance to metronidazole. The finding of differences in susceptibility patterns between human and swine strains of *C. difficile*, provides evidence that transmission between host species in this integrated population is unlikely.

Introduction

Clostridium difficile is one of the most common causes of nosocomial infections (Vonberg *et al.*, 2008). This anaerobic, Gram-positive bacterium produces spores that are both heat- and alcohol-resistant, and therefore easily transferred between patients and equipment in hospitals and nursing homes (Weir and Flegel, 2005). *Clostridium difficile* primarily affects individuals with an altered intestinal flora, following periods of prolonged antibiotic use. Patients infected with *C. difficile* experience symptoms ranging from diarrhea to severe cases of colitis that may result in death (Kuijper *et al.*, 2006).

The most common risk factor for *C. difficile* infection is antibiotic use and the risk of infection is increased if the bacteria are resistant to antimicrobial drugs (Gerding, 2004). One of the primary identifying characteristics of the NAP1/ribotype 027/toxinotype III virulent strain, responsible for recent outbreaks, is resistance to clindamycin (Fenner *et al.*, 2008; Kuijper *et al.*, 2008). Patterns of *C. difficile* infection occurring after antibiotic use have been well documented throughout the years. In the 1970s, *C. difficile* was first associated with the use of clindamycin, leading health care practitioners to decrease use (Gerding, 2004). Fluoroquinolone use began in the late 1980s and in 2001 a study linked ciprofloxacin, a commonly prescribed fluoroquinolone, to *C. difficile* infection (Yip *et al.*, 2001).

Treatment of *C. difficile* is difficult because of its direct causal relationship with antibiotic use. The two most commonly prescribed antibiotics for *C. difficile* infection are metronidazole and vancomycin. Resistance of *C. difficile* to metronidazole or vancomycin may lead to treatment failure or relapses of infection (Pituch *et al.*, 2005). Resistance to metronidazole appears to be increasing. Often an initial treatment with

metronidazole is unsuccessful and subsequent treatment with vancomycin is needed (Huang *et al.*, 2009b). However, the majority of published studies have found no resistance to either vancomycin or metronidazole among *C. difficile* isolated from clinical cases or the environment (Huang *et al.*, 2009a; Taori *et al.*, 2010).

Recent *C. difficile* studies have focused on the apparent increase in outbreaks due to NAP1/ribotype 027/toxinotype III strain infections (Drudy *et al.*, 2008; McDonald *et al.*, 2005) and an increased incidence of community-acquired infections (CDC, 2008; Huang *et al.*, 2009c). By definition, these community-acquired cases have no history of recent hospital exposure (Huang *et al.*, 2009c). Understanding the evolution and source of these strains is crucial for both treatment and prevention. One working hypothesis is that *C. difficile* may be a foodborne pathogen and this hypothesis has been supported by the isolation of *C. difficile* from both food animals and retail meat (Jhung *et al.*, 2008; Keel *et al.*, 2007; Pirs *et al.*, 2008; Rodriguez-Palacios *et al.*, 2007; Simango and Mwakurudza, 2008; Weese *et al.*, 2010; Zidaric *et al.*, 2008). An equally plausible explanation is that *C. difficile* is a ubiquitous organism and humans and food animals are exposed through a common environmental source. Investigating the antimicrobial susceptibility patterns of *C. difficile* from human and food animal populations may help us understand the association between these populations.

The objective of this study was to compare, both within and between host species, the antimicrobial susceptibility patterns of *C. difficile* strains arising from a closed and integrated human and swine population in Texas, U.S.A.

Materials and Methods

Swine composite fecal samples and human composite wastewater samples were collected from a closed, vertically integrated population in the state of Texas, previously described (Norman *et al.*, 2011; Scott *et al.*, 2005). Details on sample collection and processing and isolation of *C. difficile* have also been previously described (Norman *et al.*, 2009; Norman *et al.*, 2011; Scott *et al.*, 2005).

Antimicrobial sensitivity testing was performed on the 523 *C. difficile* strains isolated from the population using a commercially available agar diffusion Epsilon meter test (Etest®: AB Biodisk™ North America, Inc., Piscataway, NJ). Eleven different antibiotics were tested; ampicillin, chloramphenicol, tetracycline, amoxicillin/clavulanic acid, imipenem, cefoxitin, metronidazole, ciprofloxacin, clindamycin, piperacillin/tazobactam, and vancomycin.

C. difficile strains were grown anaerobically (chamber conditions: 5% hydrogen, 5% carbon dioxide, 90% nitrogen) for 48 hours on anaerobic Brucella plates (Anaerobe Systems, Walnut, CA) at 37°C. Colonies from the cultured plate were added to 1 ml of Viande-Levure (VL) broth (5g Tryptose (Becton, Dickinson, and Company (BD), Sparks, MD), 2.5 g yeast extract (BD), 2.5 g sodium chloride (VWR International, West Chester, PA), 1.2 g beef extract (BD), 0.3 g cysteine – HCl (Thermo Scientific, Rockford, IL), and 1.25 g dextrose (BD) in 500 ml of de-ionized water) to make a McFarland standard of 0.5. Anaerobic Brucella agar plates were streaked 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 2 cm apart. Plates were incubated anaerobically at 37°C and read at 48 hours, per the manufacturer's instructions (AB Biodisk™ North America, Inc.). Results were interpreted according to agar dilution

anaerobic breakpoints provided by the Clinical and Laboratory Standards Institute (CLSI) (Table 1) (CLSI, 2007). Clinical and Laboratory Standards Institute anaerobic breakpoints are not provided for vancomycin or ciprofloxacin. Vancomycin results were interpreted using the CLSI breakpoints for Gram-positive aerobes and ciprofloxacin results were interpreted based on trovafloxacin CLSI breakpoints for Gram-positive anaerobes (Table 1). Quality control strains *Bacterioides fragilis* (ATCC #25285) and *Bacterioides thetaiotaomicron* (ATCC #29741) were tested and interpreted using the recommended CLSI breakpoints.

Antimicrobial susceptibility profiles for the host species were explored using Fisher's exact test and non-parametric survival analysis. Susceptibility to each antibiotic was coded as binary and explored both between and within host species using Fisher's exact test ($p < 0.05$) (Stata SE Release 10.1). Isolates with susceptible and intermediate breakpoints were collapsed into one category and resistant isolates were placed into a second category to create binary variables. Non-parametric survival analysis was used to assess within and between host differences in the distribution of minimum inhibitory concentration (MIC) values (Stata SE Release 10.1) (Stegeman *et al.*, 2006). Log-rank tests were used to test the equality of MIC distributions and Kaplan-Meier survival curves were produced to visually compare the distributions (State SE Release 10.1). The MIC values were log transformed in order to improve the graphical interpretation of the data in the Kaplan-Meier survival curves. The lowest MIC concentrations that completely inhibited 50% (MIC_{50}) and 90% (MIC_{90}) of the strains were calculated for each of the antimicrobials. **Results**

A total of 523 *C. difficile* strains were isolated from 5,228 swine fecal and human wastewater samples (Norman *et al.*, 2011). There were 252 strains isolated from 2,936 swine fecal samples, including 175 from the farrowing barn, 14 from the nursery, 26 from breeding swine, and 37 from grower/finisher swine. There were 271 strains isolated from 2,292 human wastewater samples, including 131 from swine workers and 140 from non-workers.

Swine binary

All 252 swine strains were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, and vancomycin (i.e., 100% susceptible, 0% resistant). The majority of swine strains were resistant to cefoxitin (95.6%), ciprofloxacin (86.5%), and imipenem (58.7%) (Table 2). Interestingly, 8.3% were resistant to metronidazole. In unconditional associations, there was a significant difference in susceptibility levels between swine production groups for ciprofloxacin ($p < 0.001$) and tetracycline ($p < 0.01$). Strains from the breeding group had the highest prevalence of resistance to tetracycline and the lowest prevalence of resistance to ciprofloxacin.

Human binary

All 271 human strains were susceptible to amoxicillin/clavulanic acid, piperacillin/tazobactam, chloramphenicol, and vancomycin (i.e., 100% susceptible, 0% resistant). The majority of human strains were resistant to cefoxitin (96.3%) and ciprofloxacin (98.5%). Also, 13.3% were resistant to metronidazole. There was no significant difference in susceptibility levels between the occupational group cohorts for any of the antimicrobials.

Comparison of binary between and across host species

In unconditional analyses when comparing susceptibility between host species, there was significantly ($p < 0.05$) more resistance to ampicillin, clindamycin, and imipenem among swine strains and there was significantly ($p < 0.05$) more resistance to ciprofloxacin among human strains (Table 2). There was a significant difference in susceptibility levels across all 523 strains for chloramphenicol ($p < 0.05$), ciprofloxacin ($p < 0.001$), clindamycin ($p < 0.001$), imipenem ($p < 0.001$), and tetracycline ($p < 0.001$). Strains from the older swine production groups (grower/finisher and breeding) had a higher prevalence of resistance to chloramphenicol compared to the younger swine production groups (farrowing and nursery) and human occupational group cohorts. Interestingly, the swine breeding production group had the highest prevalence of resistance to tetracycline. There was a significant difference in susceptibility levels among seasons for clindamycin ($p < 0.05$), imipenem ($p < 0.001$), and tetracycline ($p < 0.001$) across all 523 strains. The highest percentage of resistance for clindamycin and imipenem was found in the winter and the highest for tetracycline was found in the spring.

Comparison of MIC values within and between host species

Non-parametric survival analysis was used to assess differences in the distribution of MIC values both within and between host species. Significant ($p < 0.05$) differences were found among host species with trends towards higher MIC values for imipenem, ampicillin, and clindamycin (Figure 1) in swine strains compared to human strains. Significant ($p < 0.05$) trends towards higher MIC values for ceftiofur, ciprofloxacin (Figure 2), amoxicillin/clavulanic acid, piperacillin/tazobactam, metronidazole, and tetracycline were found in human strains compared to swine strains. Key MIC values

were expressed graphically as MIC₅₀ and MIC₉₀, respectively. A significant difference was found among swine production groups for MIC values for amoxicillin/clavulanic acid (p=0.04) and clindamycin (p=0.04) (Figure 3). No significant differences (p>0.05) were found in susceptibility patterns between human occupational group cohorts.

Discussion

Decreased antimicrobial susceptibility is an important issue when treating cases of *C. difficile* in both humans and swine. The association between *C. difficile* in humans and swine is still unknown and there is evidence both supporting and refuting the possibility of transmission between species. The unique nature of the current study allowed us to explore the antimicrobial susceptibility patterns among *C. difficile* isolated from an integrated population of humans and swine with little movement in or out of the system. Previous studies have compared antimicrobial susceptibility patterns in human and swine populations; however, the populations were separate and unrelated (Debast *et al.*, 2009; Goorhuis *et al.*, 2008).

When comparing across host species, we found significantly more resistance to ampicillin, clindamycin, and imipenem among swine strains. We also found significantly more resistance to ciprofloxacin among human strains compared to swine strains. A study conducted in the Netherlands comparing swine *C. difficile* strains found similar antimicrobial susceptibility patterns for clindamycin, ciprofloxacin, metronidazole, and vancomycin with those reported for humans (Debast *et al.*, 2009). We also found similar patterns for metronidazole and vancomycin between human and swine. Another study conducted in the Netherlands also found no difference in antimicrobial susceptibility

patterns for clindamycin or ciprofloxacin between swine and humans (Goorhuis *et al.*, 2008). The type of strains we recovered may affect the antimicrobial susceptibility profiles and provide a reason for the differences in susceptibility patterns between our study and the two Dutch studies.

In addition to differences found between host species, we also found differences in susceptibility patterns between swine production groups for ciprofloxacin, tetracycline, amoxicillin/clavulanic acid, and clindamycin. We did not assess the antimicrobial usage data among these populations in the current study; however, varying rates of historical usage may be responsible for the significant differences found between host species and among swine production groups.. Alali *et al.* (2009) found that the relative odds of tetracycline resistance for *Escherichia coli* were significantly increased for chlortetracycline use in medicated feed across swine production groups in this study population (Alali *et al.*, 2009). Interestingly, the breeding group showed the highest prevalence of resistance to tetracycline and historically varying rates of chlortetracycline or oxytetracycline antibiotic treatment could explain these differences. A recent study on the use of in-feed antimicrobials in swine production found that chlortetracycline had the highest estimated use in both the nursery and grower/finisher production groups (Apley *et al.*, 2012). The few swine that entered our swine population would have been breeding boars and these introduced boars may be responsible for the observed resistance to tetracycline.

The significant differences in susceptibility patterns for clindamycin, imipenem, and tetracycline across seasons in the population (both human and swine) may also be explained by varying rates of antimicrobial use. Reduced susceptibility was observed in

the winter months for clindamycin and imipenem and in the summer months for tetracycline. This reduction may be reflective of an increase in certain conditions or illnesses that required antibiotic treatments during these seasons. . However, it should be noted that the reduced susceptibility to imipenem observed in the winter months could not be explained by differences in antimicrobial use in the swine population because carbapenems including imipenem are restricted from use in food animals.

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 MIC values. Similar results to the binary analysis were found when comparing across host species. Additionally, significantly increased MIC values were found in human strains to cefoxitin, amoxicillin/clavulanic acid, piperacillin/tazobactam, metronidazole, and tetracycline. Significant 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.

Metronidazole and vancomycin are the two most commonly prescribed antimicrobials for treatment of *C. difficile* infections in humans. The majority of clinical studies have not found resistance to either metronidazole or vancomycin (Huang *et al.*, 2009a; Taori *et al.*, 2010). All 523 swine and human strains in our study were susceptible to vancomycin. However, we found 8.3% of swine strains and 13.3% of human strains were resistant to metronidazole (Figure 4). Three studies have previously reported metronidazole resistance but at a lower prevalence (Brazier *et al.*, 2001; Pelaez *et al.*, 2002; Wong *et al.*, 1999). Clinical studies have also started to report decreasing

effectiveness of metronidazole for treatment of *C. difficile* infections (Musher *et al.*, 2005; Pepin *et al.*, 2005). One reason for the higher prevalence of metronidazole resistance found in the current study may be the source and type of *C. difficile* strains. One study has reported a greater prevalence of resistance to metronidazole in ribotype 001 strains compared to 027, 106, and several other ribotypes (Baines *et al.*, 2008).

An interesting note about our samples is that many of the metronidazole resistant strains had MIC values that were initially classified as susceptible at 24 hours. However, at 48 hours there was additional hazy growth on the plate, resulting in an MIC value interpreted as resistant. Others have also reported that certain strains appear to be heteroresistant and subpopulations show resistance to metronidazole at a slow rate (Huang *et al.*, 2010). . The bimodal distribution of the metronidazole MIC values observed in our study would support the theory of a heteroresistant population (Figure 4). Huang *et al.* also found that metronidazole resistance was lost after three passages of freezing and thawing of these isolates. We ran our Etest® strips using pure cultures from plated enrichments. When we thawed a sample of our frozen strains found previously to be metronidazole resistant, we also found that the strains were now susceptible.

There are several limitations with this study. The study design and sample collection methods do not allow us to form definitive conclusions concerning the transmission of *C. difficile* between humans and swine. The limitations of a cross-sectional study are that prevalence is the only measurable outcome and it is difficult to determine if the exposure of interest occurred before the outcome was measured. The human wastewater and swine fecal samples were aggregate samples and not collected on an individual level and results must be presented on the population level rather than the

individual level. A sample positive for *C. difficile* may be the result of one or many infected individuals. Finally little is known about the dynamics of *C. difficile* in wastewater samples. Chemicals or compounds present in wastewater may have hindered or promoted the growth, preservation, or detection of *C. difficile*.

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 community-acquired infections. Genetic sequencing of the strains from these populations would provide further information about the similarity, differences, and evolutionary history between host species. Further research is also needed to investigate the dynamics of metronidazole resistance in *C. difficile* strains.

Conclusion

The finding of differences in susceptibility patterns between host species and the finding of no difference between human occupational group cohorts provides some evidence that transmission between host species in this integrated population is less likely than within species or from point sources. The antimicrobial usage data for the host populations were not assessed in this study; however, varying rates of usage (especially for swine) may be responsible for differences seen between and within host species. The high level of resistance to metronidazole in both the swine and human wastewater samples is of concern and warrants further investigation.

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Transparency declarations

None to declare

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