including human isolates.

Multidrug Resistant and toxigenic *Clostridium difficile* isolated from commercial swine and humans

Thakur, S*,(1), Putnam, M.(1), Fry, P.(2), Abley, M.(2), Gebreyes, W.(2)

(1)Department of Population Health and Pathobiology, College of Veterinary Medicine, North Carolina State University, Raleigh, North Carolina 27606
(2)Department of Veterinary Preventive Medicine, College of Veterinary Medicine, The Ohio State University, Columbus, OH 43210

corresponding author: sthakur@ncsu.edu

Abstract

This study was conducted to compare *C. difficile* population in commercial swine with those causing *C. difficile* associated diarrhea (CDAD) cases in humans. Fecal samples were collected from sows (eight per farm) and piglets (30 per farm) in eight farms in North Carolina (n=5) and Ohio (n=3) representing a total of 68 sows and 251 piglets. In addition, 33 *C. difficile* isolates were collected from CDAD cases in humans from the NC region. *C. difficile* isolates were tested for their susceptibility to a panel of six antimicrobials. PCR was used to detect genes coding for enterotoxin A (*tcdA*), cytotoxin B (*tcdB*) and the binary toxin (CDT). We detected significantly higher piglet prevalence in Ohio (87.5%) than North Carolina (64%) ($P < 0.001$). The frequency of resistance was the highest to ciprofloxacin including piglets (91.3%), sows (93.8%) and humans (100%). Majority of the isolates were positive for virulence genes encoding the enterotoxin (*tcdA*), cytoxoin (*tcdB*) and the binary toxin (*cdtB*). We found different variants of the toxins A and B among the human isolates including A^+B^+, A^-B^+ and A^-B^- profile accounting for 81.8% (n=27), 12% (n=4) and 6% (n=2), respectively. We identified different toxin profile among pigs and humans which indicates other sources of transmission. The findings strongly show that toxigenic strains of *C. difficile* are common in piglets and contribute to high mortality during farrowing.

Introduction

C. difficile is an important pathogen in food animals and is responsible for causing colitis in neonatal piglets (Songer and Anderson, 2006). Recent reports on identification of pathogenic strains of *C. difficile* in humans, including Toxinotype V commonly seen in pigs highlights the possible transmission of these strains from pigs to humans (Rupnik et al., 2008). However, there are very limited studies conducted to determine the epidemiology and potential significance of toxigenic and multi-drug resistant (MDR) *C. difficile* strains in pigs and their relation to strains isolated from humans. Prompted by the lack of *C. difficile* data in pigs, the main objective of this study was to determine the prevalence of *C. difficle*, assess the occurrence of MDR phenotypes and determine any relationship with isolates from humans.

Materials and methods

We collected pig fecal samples from a total of eight different farms, including five from North Carolina and three from the Ohio states. A total of 30 piglets (1-7 days age) and eight sows were sampled at every farrowing farm. Every pig was sampled thrice starting at the farrowing unit with subsequent sampling at nursery and finishing farms. The 33 *C. difficile* isolates from human were collected from clinical CDAD patients in North Carolina in the same time period as the swine sampling. The MIC was determined against a panel of six antimicrobials using the Epsilometric test including ampicillin, ciprofloxacin, erythromycin, metronidazole, tetracycline and vancomycin. We used the breakpoint levels used in a previous study for the antimicrobials (Zheng et al., 2007). Amplification of the housekeeping gene *tpi* and the toxins genes was done using specific primers as described previously (Lemee at al., 2004). The frequency of antimicrobial resistance patterns and MIC levels comparison between isolates from pigs and humans were compared using the χ^2 test (Minitab Inc. PA, USA) and Fisher's exact two-tailed test when applicable. Results with a Type I error of $p \leq 0.05$ was considered statistically significant.

Results

A total of 251 piglets and 68 sows were sampled in this study from five farms in North Carolina (155 piglets and 44 sows) and three farms in Ohio (96 piglets and 24 sows). The overall *C. difficile*

prevalence in piglets was 73% (n=183) with significantly higher prevalence of 87.5% (n=84) in Ohio than North Carolina: 64% (n=99) ($P < 0.001$). *C. difficile* isolates from pigs and humans exhibited resistance to four out of the six antimicrobials tested (Table 1). Overall, the frequency of resistance to ciprofloxacin (Cip^R) was the highest irrespective of the source or the region with 91.3% frequency among isolates from piglets and 94% from sows ($p>0.05$). Resistance to gatifloxacin was observed only in *C. difficile* isolates from humans (21%). All the *C. difficile* isolates in this study were susceptible to metronidazole and vancomycin. The MIC_{50} and MIC_{90} to ciprofloxacin was > 32 µg/mL for isolates from pigs and humans. The MDR pattern Cip^R-Ery^R-Tet^R was the predominant pattern in pigs represented by 19.5% (n=42) of the total isolates. The predominant MDR pattern in humans was the Cip^R-Ery^R-Gat^R-Lev^R pattern (21%) followed by the Cip^R-Ery^R-Lev^R pattern (6%).

We detected toxins A (65%) and B (84%) and the binary toxin coding genes (77%) in piglets and sows. Human isolates had a different profile with 81% of the isolates positive for *tcd*A, 94% for *tcd*B and 24% for *cdt*B. Further analysis revealed a combination of four different toxin encoding genes that were predominant. The toxin gene profile coding for $A^+B^+CDT^+$ was predominant found in 59% (n=127) of the swine isolates (Table 2). Thirty eight isolates (18%) exhibited the $A^-B^+CDT^+$ profile while 33 (15.3%) isolates tested negative for all the toxins tested. On comparing the two geographic regions of sampling, a significantly higher number of *C. difficile* isolates from piglets under the Ohio farms (n=28) had the above profile ($P < 0.001$). The majority (63.6%) of human isolates had the profile $A^+B^+CDT^-$. Other human profiles included $A^+B^+CDT^+$ (18.1%) and $A^-B^-CDT^+$ (6%) and toxin negative $A^-B^-CDT^-$ (6%).

Discussion

Clostridium difficile is an important pathogen and is responsible for causing enteric diseases in pigs. The high prevalence of *C. difficile* in piglets in this study (73%) in pigs was not surprising as CDAD is a known cause of neonatal enteritis (Songer and Anderson, 2006). Previous studies have reported *C. difficile* prevalence in piglets from 25.9% to 49.5% and the findings in this study are in agreement with these reports (Harvey et al., 2008; Brazier et al., 2008). Other than a single pig that was positive at nursery, none of the remaining pigs were positive at nursery and finishing stages of production. Previous studies have also reported similar findings with significantly higher prevalence in piglets than adult pigs at nursery or finishing levels (Harvey et al., 2008). There is a dearth of information highlighting the antimicrobial resistance profile of *C. difficile* isolated from pigs. Antimicrobial resistance to ciprofloxacin was observed at the highest concentration tested (32 µg/mL) in majority of the isolates from piglets (91.3%) and sows (94%). The results are in accordance with other studies that have reported high frequency of resistance to ciprofloxacin in *C. difficile* isolates from different sources including pigs and humans (Razavi et al., 2007; Harvey et al., 2008). There was no indication of elevated MIC for metronidazole and vancomycin, the two choices of drugs for treating infections in humans. These results are in accordance with previous reports (Zheng 2007; Razavi 2007; Brazier et al., 2008). The detection of specific resistance patterns associated with geographic location of sampling in this study was interesting. This may indicate that specific *C. difficile* strains are circulating in specific locations. Also, the isolation of *C. difficile* isolates with similar resistance patterns from the sow and piglets may imply a direct transmission of *C. difficile* from the sow to the piglets. However, this study was conducted in limited number of farms and may not have external validity. The detection of resistance to important antimicrobials is concerning and should be studied in more detail using representative samples.

The $TcdA^+B^+CDT^+$ toxin profile was the predominant swine gene profile represented by 59% of the total isolates. However, the detection of toxin negative *C. difficile* strains from the feces of pigs in this study (15.3%) is an important finding and necessitates the importance of pathogen isolation and not simply relying on toxin detection to confirm its presence. This is the first study to report the $TcdA^-B^+CDT^+$ gene profile from pigs and is an important finding. *C. difficile* strains with the above toxin profile have been reported to cause outbreaks and clinical cases in humans and isolated with increasing frequency from both infants and elderly (Drudy et al., 2006; Shin et al., 2008). The prevalence of binary toxin coding genes was high for swine with 77% (n=166) of the isolates positive for the *cdtB* gene. Previous reports have shown a higher prevalence of this toxin in piglets ranging from 78.4% to 83% (Rupnik et al., 2008; Zidaric et al., 2008). Binary toxin has also been associated with community acquired-CDAD cases in humans (Barbut et al., 2005). However, in this study, human isolates positive for CDT (24%) was

significantly lower compared to swine isolates. The TcdA$^-$B$^+$ toxin profile was represented by four human isolates and have been isolated with increasing frequency from infants as well as the elderly (Shin et al., 2008). The high prevalence of this toxin in pigs can have serious implications for the workers who are in close association with them on farms.

Conclusions

In conclusion, we identified MDR and virulent strains of *C. difficile* circulating in swine populations at the farrowing level in two states in the US which were different from those observed in humans. The detection of fluoroquinolone and macrolide resistance in these isolates is concerning due to the use of these drugs in treating infectious diseases in humans. Our findings highlight the importance of conducting further detailed studies to better understand the epidemiology of *C. difficile* in swine and their environment and to determine other sources of community acquired CDAD in humans.

References

BARBUT F., DECRE D., LALANDE V., BURGHOFFER B., NOUSSAIR L., GIGANDON A., ESPINASSE F., RASKINE L., ROBERT J., MANGEOL A., BRANGER C., PETIT J., 2005, Clinical features of *Clostridium difficile* associated diarrhoea due to binary toxin (actin-specific ADP-ribosyltransferase)-producing strains. J. Med. Microbiol. 54, 181-185.

BRAZIER J. S., RAYBOULD R., PATEL B., DUCKWORTH G.,PEARSON A., CHARLETT A., DUERDEN B. I., HPA REGIONAL MICROBIOLOGY NETWORK, 2008, Distribution and antimicrobial susceptibility patterns of *Clostridium difficile* PCR ribotypes in English hospitals, 2007-08. Euro. Surveill. 13, pii, 19000.

DRUDY D., QUINN T., O'MAHONY R., KYNE L., O'GAORA P., FANNING S., 2006, 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. 58, 1264-1267.

HARVEY R. B., NORMAN K. N., SCOTT H. M., HUME M., ANDREWS K., 2008, Prevalence of Clostridium difficile in an integrated swine operation, in *Anaerobe*. The 9th Biennial congress of the Anaerobe Society of the Americas, 24-27.

LEMEE L, DHALLUIN A, TESTELIN S, MATTRAT M., MAILLARD K., LEMELAND J., PONS J., 2004, Multiplex PCR targeting *tpi* (triose phosphate isomerase), *tcdA* (Toxin A), and *tcdB* (Toxin B) genes for toxigenic culture of *Clostridium difficile*. J. Clin. Microbiol. 42, 5710-5714.

RAZAVI B., APISARNTHANARAK A., MUNDY L. M., 2007, *Clostridium difficile*: emergence of hyperviluence and fluoroquinolone resistance. Infection. 35, 300-307.

RUPNIK M., WIDMER A., ZIMMERMANN O., ECKERT C., BARBUT F., 2008, *Clostridium difficile* toxinotype V, ribotype 078, in animals and humans. J. Clin. Microbiol. 46, 2146.

SHIN B. M., KUAK E. Y., YOO H. M., KIM E. C., LEE K., KANG J., WHANG D. H., SHIN J., 2008, Multicentre study of the prevalence of toxingenic *Clostridium difficile* in Korea: results of a retrospective study 2000-2005. J. Med. Microbiol. 57, 697-701.

SONGER. J. G., ANDERSON M. A., 2006, *Clostridium difficile*: an important pathogen of food animals. Anaerobe. 12, 1-4.

ZHENG L, CITRON DM, GENHEIMER CW, SIGMON S. F., CARMAN R. J., LYERLY D. M., GOLDSTEIN E. J., 2007, Molecular characterization and antimicrobial susceptibilities of extra-intestinal *Clostridium difficile* isolates. Anaerobe. 13, 110-114.

ZIDARIC V., RUPNIK M., AVBERSE, J., ZEMLJIC M., JANEZIC S., PIR T., OCEPEK M., 2008, Prevalence and diversity of Clostridium difficile in poultry, pigs and calves, in *Anaerobe*. The 9th Biennial congress of the Anaerobe Society of the Americas, 24-27.

Table 1. Antimicrobial resistance frequency of *C. difficile* at different MIC from piglets and sows.

Antimicrobial[a] (Dilution Range µg/mL; Breakpoint)	Source[c]	MIC range[d] (µg/mL)	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	Resistance[e] n(%)
Ampicillin (0.016-256; 2)	Sow	0.032-1.5	0.5	0.75	0
	Piglet	0.0016-2	0.75	1	5 (2.7)
	Humans	0.016-2	0.75	1.5	1 (3)
Ciprofloxacin (0.002-32; 8)	Sow	0.38->32	>32	>32	30 (94)
	Piglet	0.047->32	>32	>32	167 (91.3)
	Humans	>32	>32	>32	33 (100)
Erythromycin (0.016-256; 2)	Sow	0.047->256	0.75	>256	11 (34.4)
	Piglet	0.032->256	1	>256	70 (38.3)
	Humans	0.094->256	1	>256	15 (45.4)
Tetracycline (0.016-256; 4)	Sow	0.023-64	2	64	10 (31.3)
	Piglet	0.016-48	4	16	84 (46)
	Humans	0.016-16	0.016	0.13	1 (3)

[a]For every antimicrobial, the dilution range in µg/ml and resistance breakpoint level is shown, [b]Represents the number of *C. difficile* isolates tested from piglets (n=183), sows (n=32) and humans (n=33), [c]Indicates the MIC range detected for *C. difficile* isolates tested; [d] Indicates number (%) of *C. difficile* isolates exhibiting resistance to an antimicrobial.

Table 2. Comparison of *C. difficile* toxin gene profile isolated from pigs from the two regions

Source[a]	Region	Toxin Gene Profile n(%)[b]			
		$A^+B^+CDT^+$ [c]	$A^-B^+CDT^+$	$A^-B^-CDT^-$	$A^+B^+CDT^-$
Piglet	North Carolina	59(59.6)	25(25.3)	2(2)	10(10)
	Ohio	46(54.8)	7(8.3)	28(33.3)	3(3.5)
Sow	North Carolina	16(72.7)	5(22.7)	0	0
	Ohio	6(60)	1(10)	3(30)	0
Humans	North Carolina	6(18.1)	2 (6)	2 (6)	21 (63.6)
Overall		133(53.6)	40(16.1)	35(14.1)	34(13.7)

[a]Represents the number of *C. difficile* isolates tested from piglets (n=183), sows (n=32) and humans (n=33), [b] Indicates the number (%) of *C. difficile* isolates with a particular toxin gene profile, [c]Represents the toxins encoded by the genes including toxins A (TcdA) and B (TcdB) and the binary toxin (CDT).