RESEARCH REPORT
PUBLIC HEALTH/WORKER SAFETY

Title: Epidemiological survey to determine the prevalence of Clostridium difficile in swine in an integrated swine operation" **NPB #06-156**

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Abstract

The objectives of this study were to compare the prevalence of *Clostridium difficile* (Cd) among different age and production groups of swine in a vertically integrated swine operation in Texas in 2006 and to compare our isolates to other animal and human isolates. Results are based on 131 Cd isolates from 1008 swine fecal samples and pork trim samples (overall prevalence of 13%). The prevalence (number positive/number tested in production type) of Cd was different between the groups ($P \leq 0.001$), and was highest among farrowing barn inhabitants (predominantly piglets, but also included lactating sows and effluent) at 36.5% (95/260), followed by 8.2% (10/122) for nursery, 6.5% (4/62) for pork products, 3.9% (15/382) for grower-finisher, and 3.8% (7/182) for breeding boars and sows. Of the 131 isolates, 122 were positive by PCR for both toxins A (*tcd*A) and B (*tcd*B) genes, 129 isolates harbored a 39 base pair deletion in the *tcd*C gene, 120 isolates were toxinotype V, and all 131 of the isolates were positive for the *cdt*B binary toxin. All isolates were resistant to cefoxitin, ciprofloxacin, and imipenem, whereas all were sensitive to metronidazole, piperacillin/tazobactam, amoxicillin/clavulanic acid, and vancomycin. The majority of isolates were resistant to clindamycin; resistant or intermediate to ampicillin; and sensitive to tetracycline and chloramphenicol. There was an increased

($P \leq 0.001$) number of isolates for the timeframe of September to February compared to March to August. Cd most commonly originated among farrowing barn production types (primarily piglets) and not in grower/finisher production.

Introduction

Since 2003, the incidence and severity of disease associated with toxigenic *Clostridium difficile* (Cd) have increased in hospitals in North America [1,2]. Indications are that this increase may be due to emergence of a new strain, (North American pulsed field gel electrophoresis 1, [BI/NAP1]) of toxigenic Cd that has increased resistance, virulence, or both. Health care officials are concerned because the emergent strain can be community-acquired as well as hospital-acquired [2]. The origins of this epidemic strain have yet to be determined. Animals can be colonized and/or infected with various strains of Cd including the above-mentioned NAP1 [3]. However, the strains predominantly isolated from food animals belong to pulsed field group NAP7 [3]. Researchers have isolated Cd from food animals and from retail beef, turkey, and pork, and some speculate that Cd could be transmitted to humans through food sources [3-5]. No epidemiologic information is available on the prevalence and the genetic make-up of toxigenic Cd from healthy swine in commercial operations.

Objective

The objective of the present study is: a) to compare Cd prevalence in different age and production groups of swine in an integrated swine operation in Texas [6-8], b) to phenotypically and genotypically characterize our Cd isolates, and c) to compare our isolates to other animal and human Cd isolates.

Materials and Methods

Composite fecal samples (representing a minimum of 10 pigs/sample) and pre-lagoon effluent were collected monthly over a 12-month period (February 2006 to January 2007) from 13 separate farms at three different geographical locations (up to 200 km apart) in Texas. The farms were comprised of five farrow-to-finish units, six grower-finisher units, one purebred boar quarantine unit, and one slaughter plant (pork trim and kill floor effluent). Production groups tested were breeding boars and sows, lactating sows, nursing piglets, weaned

nursery pigs, and grower and finisher pigs. We sampled the farrowing, finisher, and purebred boar production groups at a disproportionally higher frequency than other production groups because nursing piglets can have a high colonization rate of Cd, Cd-positive finisher pigs could represent a risk factor in transfer of Cd to pork (finished food product), and purebred boars often originate from high-health herds with a history of antibiotic usage. Each composite fecal sample consisted of 10 partial fecal pats per pen (approximately 30 ml total) collected into 50 ml conical tubes. Fecal samples were collected from pens of asymptomatic, clinically healthy swine. Immediately after collection, samples were stored on ice and transported within 4 h to the Food and Feed Research Unit, USDA, College Station, TX.

Cultivation of Cd was performed by use of the techniques described by Rodriguez-Palacios et al., [3]. Briefly, this consisted of alcohol shock, enrichment media, concentration by centrifugation, and incubation in an anaerobic chamber. Presumptive diagnosis consisted of presence of colonies morphologically similar to Cd, L-proline aminopeptidase activity (Pro Disc, Remel, Lenexa, KS), and biochemical characterization (Rapid ID 32A, bio-Merieux, Durham, NC).

PCR was used to determine the presence of toxin A&B genes (*tcd*A and *tcd*B), for the *tcd*C regulatory gene deletion, for toxinotyping, and for the presence of *cdt*B binary toxin gene by using the techniques utilized by the Centers for Disease Control and Prevention (CDC), Atlanta, GA [9-11].

Each Cd isolate was tested for its antibiotic susceptibility to eleven antibiotics (ampicillin, chloramphenicol, tetracycline, amoxicillin/clavulanic acid, imipenem, cefoxitin, metronidazole, ciprofloxacin, clindamycin, piperacillin/tazobactam, and vancomycin) by use of the Etest® (AB Biodisk™ North America, Inc., Piscatway, NJ) according to the manufacturer's recommendations. Minimum inhibitory concentrations (MIC) for antibiotics and interpretive values are presented in Table 1. Results were interpreted according to standard criteria except MIC's for ciprofloxacin were based on values for trovafloxacin, whereas vancomycin interpretation was based on MIC's reported for Gram positive aerobes. Quality control strains, *Bacterioides fragilis* (ATCC #25285) and *B. thetaiotaomicron* (ATCC #29741) were tested using recommended breakpoints for MIC's [12].

Statistical analyses (in XTLOGIT procedure:Stata SE Release 10.1, Stata Corp., College Station, TX) were those appropriate for time-series cross-sectional data. The outcomes were considered as binary (i.e., presence/absence of Cd per samples, subcategories per isolate and sample), and mixed logistic (fixed and random effects) methods were employed to adjust for the dependence of response both within cluster (e.g. location or unit) as well as over time (12 monthly samples) using location as a nuisance parameter (i.e., treated as a random variance component) [13].

Results

There were 1008 samples collected and tested from the following production and age groups of swine or pork: farrowing barns (lactating sows, piglets, and effluent), nursery (weaned piglets), pork trim, grower/finisher pigs and breeding sows/boars (Table 2). Although nursing piglets, lactating sows and farrowing barn effluent were cultured separately, the authors were of the opinion that all three were intertwined and that a positive Cd sample from one source was reflective of what would be found in the other sources. Therefore, for analysis, all three were combined into a production group referred to as "farrowing barn". Likewise, results from "grower" and "finisher" pigs were combined into "grower/finisher" groups. Table 2 illustrates the differential culture sampling proportions that were applied based on the expected variation in prevalence by production group. Of 131 Cd-positive samples, the prevalence of Cd (number of composite sample Cd-positive/number of Cd-tested) differed significantly ($P \leq 0.001$) between production groups as follows: a) 36.5% for farrowing barns, b) 8.2% for nursery, c) 6.5% for pork trim, d) 3.9% for grower finisher, and e) 3.8% for sows and boars.

The PCR results for toxins A and B showed that 122 isolates were positive for both A and B toxin genes, 2 isolates were negative for toxin A and positive for toxin B, and 7 isolates were negative for both toxins A and B. Furthermore, 129 of the isolates harbored a 39 bp deletion in the *tcd*C regulatory gene, all 131 of the isolates tested positive for the *cdt*B binary toxin gene, and 120 of the isolates (including 4 pork trim isolates) were toxinotype V. The 2 toxin A-B+ isolates had no base pair deletions in the *tcd*C gene. Toxinotypes were not determined for 11 isolates. They were: 7 toxin A-B- isolates, 2 toxin A-B+ isolates, and 2 toxin A+B+, *cdt*B+ (not toxinotype V) isolates.

The multivariate model tested the following variables: production group, month, season (winter; [December, January, February] spring; [March, April, May], summer; [June, July, August], fall; [September, October, November]), and geographical location. The mixed model logistic regression found that the main effects for production type were significant ($P \leq 0.001$) in predicting the presence of Cd (Table 3). Month, season, or geographical location was not significant ($P \geq 0.05$). Season was not significant to the model when the four seasons were included; however, when fall and winter were collapsed into a category (September to February) and spring and summer were collapsed into a second category (March to August), they were significant ($P \leq 0.001$). During the cooler months, 16.2% of samples tested were positive for Cd, whereas 10.3% of samples tested during the warmer months were positive (mean prevalence of 13.2% for 1008 samples). Interactive effects between production group and season were also significant ($P \leq 0.05$) (Table 3). The intercept only model suggested that 20% of the total variance was attributed to geographical location ($P \geq 0.05$). In the final model, only 8.5% of the total variance was attributed to location ($P \geq 0.05$) showing that most of the variation in location was explained by the production groups at those locations.

CLSI MIC values and interpretive results [12] along with our sensitivity interpretations for this study are presented in Table 1. All (100%) of the Cd isolates were resistant to cefoxitin, ciprofloxacin, and imipenem, whereas 92% were resistant to clindamycin. Fifty-three percent were intermediate, 36% resistant and 11% sensitive to ampicillin. All (100%) isolates were sensitive to metronidazole, vancomycin, pipercillin/tazobactam combination, and amoxicillin/clavulanic acid combination; 98% were sensitive to chloramphenicol; and 90% were sensitive to tetracycline. MIC values for quality control strains *B. fragilis* (ATCC #25285) and *B. thetaiotaomicron* (ATCC #29741) were well within published guidelines [12].

Discussion

It is evident in this study that suckling piglets in the farrowing barn contributed the largest number of isolates ($P \leq 0.001$) compared to other production groups. This is in agreement with other reports in that young animals such as piglets and calves appear to have increased Cd carriage compared to more mature animals [3,14,15].

PCR results for the *tcd*A and *tcd*B genes showed that the majority (122/131) of the isolates were positive for both toxin genes. However, not all strains of Cd produce toxins on a predictable basis. For example in one study with human and animal isolates, 100% of toxinotype V Cd produced toxins A and B [15]. In that study, the toxinotype V strains had greater toxin production than non-epidemic toxinotype 0 isolates, but less than epidemic toxinotype III isolates [15]. In another study, 19 of 31 isolates from dairy calves produced toxins A and B while the remaining 12 isolates were deficient in either toxin A or B [3]. Although Cd may be present with no toxin production, toxin production can be detected in feces without the ability to cultivate the organism. For example, toxins A and B were detected in feces of 85 of 278 calves mentioned in the above study, yet only 31 Cd organisms were isolated from the same 278 calves [3]. In the present study, toxinotyping results showed that 120 of our isolates (including 4 pork trim isolates) were toxinotype V. These results are consistent with other studies that have shown toxinotype V, PCR ribotype 078 to be the predominant strain in pigs, calves, and pork [3,4,15,16]. In our study, 7 of the isolates were toxin A-B- and *cdt*B+ (binary toxin). Although we were unable to determine a toxinotype, similar toxin patterns have been described for toxinotypes XIa and XIb [17]. Two of our isolates were A-B+ and *cdt*B+, and although no toxinotype was ascribed, toxin patterns would suggest that V-like toxinotype could be a likely candidate [17]. The remaining 2 isolates were A+B+ and *cdt*B+, but had no bp deletions in the *tcd*C gene. Restriction patterns were similar to those of toxinotype XXIV [17].

It is not unusual to have seasonal variations for the presence of enteric bacterial pathogens in domestic animals; however, most studies have shown an increase in prevalence during the warmer months. For example, cattle shed *Campylobacter* spp. more heavily in the spring and autumn [18], peak human infections of *Campylobacter* spp. occur in mid-June [19], and ruminants have the highest fecal prevalence of *E. coli* O157:H7 in the summer months [20]. Somewhat in contrast to our study and although the study did not cover a 12-month period, Rodriquez-Palacios et al. [3] reported that dairy calves were more likely to yield a positive Cd toxin test result in May-July compared to August. In our study, there was an increased number of Cd isolates in the cooler months compared to the warmer months. The authors are unaware of any changes in production practices in

winter versus summer that could affect Cd colonization. Pigs were kept in pens that were open and exposed to the elements, and it is unknown if exposure to the colder temperatures, wind, and rain in the winter months could increase the pigs susceptibility to colonization by Cd. Weather can play a part in colonization of some bacteria. For example, *C. perfringens* is reported to have an increased prevalence in marine mammals and invertebrates during the wet season [21].

Of the eleven antibiotics tested, it is interesting to note that isolates were resistant to four of the antibiotics, sensitive to six antibiotics, and intermediate to one. Because a large range of antibiotics were tested, our results are not directly comparable to that of other Cd studies. In a study of Cd (predominately 078, 077, and 017 ribotypes) in dairy calves, all 30 Cd isolates were susceptible to metronidazole and vancomycin, and 73% were resistant to clindamycin and levofloxacin [3]. These results are similar to the antimicrobial resistance patterns of 12 Cd (predominately PCR ribotype 31, toxinotype III) isolated from meat in Canada in which all were susceptible to metronidazole and vancomycin and all resistant to levofloxacin and clindamycin [5]. In our study, 90-100% of isolates were sensitive to metronidazole, vancomycin, pipercillin/tazobactam, amoxicillin/clavulanic acid, chloramphenicol, or tetracycline. Results indicate that 100% and 92% of our isolates were resistant to ciprofloxacin and clindamcyin, respectively. When comparing human and animal toxinotype V Cd isolates, it was noted that most resistance patterns overall were similar [15]. In that study, 88% of bovine isolates were sensitive to clindamycin, whereas only 0% or 9% of porcine or human isolates, respectively, were sensitive. In studies with human isolates, non-epidemic (non-BI/NAP1) strains of Cd were 100% resistant to ciprofloxacin, 33% were resistant to moxifloxacin and gatifloxacin, and 50% were resistant to levofloxacin and clindamycin [22]. In contrast, BI/NAP1 strains were resistant to ciprofloxacin, moxifloxacin, gatofloxacin, and levofloxacin, and susceptible to clindamycin. All strains were sensitive to metronidazole and vancomycin [22]. On the basis of the antibiotic sensitivity results of the present study, it appears that our isolates are somewhat less resistant to antibiotics than some human isolates [1,15,22].

Conclusions and Future Directions:

In this study, 72% of Cd isolates came from the farrowing barns arising from 5 farrow-to-finish farms and piglets were the predominant source of those isolations, demonstrating that they were the production group with the highest carriage rate ($P \leq 0.001$) of Cd. The majority of our isolates were toxinotype V, which is similar to that reported for swine. Geographical location, month, or individual season did not appear to influence the isolation rate of Cd ($P > 0.05$); however, there was a greater ($P \leq 0.001$) number of isolates from the fall-winter timeframe than spring-summer. Antimicrobial resistance of our isolates appeared to be similar to other veterinary isolates, although ours tended to be less resistant than that reported for human Cd isolates. While market age and breeding animals were sampled on a disproportionate basis compared to other production groups, these older animals had a very low prevalence rate of Cd. If Cd can be transmitted to humans via the food chain, then lowered prevalence in older animals could possibly be a factor for decreased transmission risk for Cd (as compared to the younger-aged cohorts).

We are in the process of further characterization of our isolates. Future work includes PCR ribotyping and pulsed field gel electrophoresis (PFGE). We will submit our PFGE digital images from our swine isolates to the CDC to compare to other veterinary and human isolates in their data base. Additionally, there is a human population on the same premises as the swine study and in the future we intend to assay human wastewater samples for Cd from those locations (NPB Grant #-08-188). Any human isolates from our future work will be characterized and handled in the same manner as swine isolates and appropriate comparisons reported.

Lay Interpretation

In recent years, there has been increased incidence and severity of human disease associated with the bacterium *Clostridium difficile* (Cd). It appears that epidemic disease is being caused by newly emerging strains of Cd. No one knows where the new strains came from, but several human disease strains have been compared to strains found in swine. On that basis, some researchers speculate that the new strains may have come from pigs or could be food-associated (i.e., came from pork). We proposed this study to determine how frequently pigs (across production and age groups) are colonized by Cd and how pig isolates compare to other animal and

human Cd isolates. We isolated 131 Cd from predominantly suckling piglets, but very few from finisher or adult swine. The majority of isolates occurred during the cold months. Our pig isolates did not appear to have as much resistance to antibiotics as human isolates do. We determined that our pig isolates are not the human epidemic strain. Although these pig strains can colonize humans and cause disease, they are not the most common strains seen in human disease. We found 4 isolates in pork trim used to make sausage and these strains were of the same type as found in our pigs and also the same as most commonly reported for meat. Because of the low carriage rate of Cd by market age pigs and the fact that Cd had a low frequency in meat, we conclude that pork is a low risk for transmission of Cd into the food chain.

References

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Table 1. *Clostridium difficile* antibiotic sensitivities: minimum inhibitory concentrations (MIC), interpretive categories, and interpretive results

Antibiotic	MIC S[a]	I	R	Results/131 tested
Amoxicillin-clavulanic acid	≤4/2	8/4	≥16/8	131S
Ampicillin	≤0.5	1	≥2	14S, 70I, 47R
Cefoxitin	≤16	32	≥64	131R
Chloramphenicol	≤8	16	≥32	128S, 1I, 2R
Clindamycin	≤2	4	≥8	10I, 121R
Imipenem	≤4	8	≥16	131R
Metronidazole	≤8	16	≥32	131S
Piperacillin-tazobactam	≤32/4	64/4	≥128/4	131S
Tetracycline	≤4	8	≥16	118S, 8I, 5R
Ciprofloxacin[b]	≤2	4	≥8	131R
Vancomycin[b]	≤4	8-16	≥32	131S

[a]Sensitive, Intermediate, and Resistant MIC values from CLSI [12]

[b]Ciprofloxacin interpretation based on MIC for trovafloxacin; vancomycin interpretation based on MIC for Gram positive aerobes

Table 2. *Clostridium difficile* prevalence (number positive/number tested) by production group in an integrated swine operation.

Production Group	Positive/Tested (%)
Farrow	95/260 (36.5)
Nursery	10/122 (8.2)
Pork Trim	4/62 (6.5)
Grow/Finish	15/382 (3.9)
Breeding Sows/Boars	7/182 (3.8)
Totals:	131/1008 (13.2)

Table 3. Coefficient and Odds Ratio results from the multivariable model testing the main effects and interaction of season and production groups for predicting *Clostridium difficile* in swine.

Risk factor	P-value (LR test d.f.)	Category	Coefficient	Adjusted Odds Ratio	OR 95% Confidence Interval
Intercept			-0.03	-	-
Season	<0.001 (1 d.f.)	winter (referent category)	-	-	-
		summer	-1.13	0.32	0.19--0.56
Swine production group	<0.001 (3 d.f.)	farrowing (referent category)	-	-	-
		grower/finisher	-3.47	0.03	0.01--0.08
		nursery	-2.62	0.07	0.02--0.22
		breeding	-3.00	0.05	0.02--0.15
Interaction of Season by Swine production group	0.049 (3 d.f.)	farrowing – winter	-	-	-
		farrowing – summer	-	-	-
		grower/finisher – winter	-	-	-
		grower/finisher – summer	1.38	3.98	1.12--13.07
		nursery – winter	-	-	-
		nursery – summer	1.42	4.12	0.98--17.32
		breeding – winter	-	-	-
		breeding – summer	0.54	1.72	0.34--8.73

Appendix A—Publications Generated

1. Harvey RB, Hume ME, Norman KN, Scott HM, Andrews K, Martin JD, et al. *Clostridium difficile* prevalence in an integrated swine operation in Texas. Proceedings of 2007 Allen D. Swine Conference, University of Minnesota, 2007;34(Suppl):28.

2. Norman KN, Scott HM, Harvey RB, Hume ME, Andrews K, Martin JD. Comparison of the prevalence and genomic characteristics of *Clostridium difficile* in an integrated swine operation in Texas in 2006. Proceedings of the Annual Meeting of the Conference of Research Workers in Animal Diseases, 2007; p. 137.

3. Harvey RB, Norman KN, Scott HM, Hume ME, Andrews K. Prevalence of *Clostridium difficile* in an integrated swine operation. Proceedings of the 9th Biennial Congress of the Anaerobe Society of the Americas, 2008; p. 151.

4. Norman KN, Harvey RB, Scott HM, Hume ME, Andrews K, Brawley AD. Varied prevalence of *Clostridium difficile* in an integrated swine operation. Anaerobe (Submitted 4/7/09).

5. Harvey RB, Norman KN, Hume ME, Andrews K, Scanlan CM, McReynolds JL, Scott HM. *Clostridium difficile* in mixed populations of animals and humans. Proceedings of 26th International Congress of Chemotherapy and Infection, June 18-21, 2009, Toronto, Canada.

6. Harvey RB. *Clostridium difficile* in cattle and swine. Proceedings of 2009 Joint Annual Meeting ADSA, CSAS, ASAS, July 12-16, 2009, Montreal, Canada.

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8. Norman KN*, Scott HM, Harvey RB, Norby B, Hume ME, Andrews K. Comparison of the prevalence and genotypic characteristics of *Clostridium difficile* isolated from various production groups in a vertically integrated swine operation. Proceedings of XII Conference of International Society of Veterinary Epidemiologists and Economics, August 10-14, 2009, Durban, South Africa.

9. Norman KN*, Scott HM, Harvey RB, Norby B, Hume ME, Andrews K. Comparison of the prevalence and genotypic characteristics of *Clostridium difficile* in a closed and integrated human and swine population in Texas. Proceedings of the XII Conference of International Society of Veterinary Epidemiologists and Economics, August 10-14, 2009, Durban, South Africa.

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