Clinical microbiology

Varied prevalence of *Clostridium difficile* in an integrated swine operation


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**A B S T R A C T**

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 < 0.001), and was highest among suckling piglets at 50.0% (61/122), followed by 23.8% (34/143) for lactating sows and effluent from the farrowing barn, 8.4% (10/119) for nursery, 6.5% (4/62) for pork products, 3.9% (15/382) for grower-finisher, and 3.9% (7/180) for breeding boars and sows. Of the 131 isolates, 122 were positive by PCR for both toxins A (*tcdA*) and B (*tcdB*) genes, 129 isolates harbored a 39 base pair deletion in the *tcdC* gene, 120 isolates were toxinoftype V, and all 131 of the isolates were positive for the binary toxin gene *cdtB*. All isolates were resistant to cefoxitin, ciprofloxacin, and imipenem, whereas all were sensitive to meropenem, 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 < 0.001) number of isolates for the timeframe of September to February compared to March to August.

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**1. 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, (*Restriction endonuclease analysis type BI, North American pulsed field gel electrophoresis pattern 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. The objectives of the present study were 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.

**2. Materials and methods**

**2.1. Sample collection**

Composite fecal samples (representing a minimum of 10 pigs/sample) and pre-lagoon effluent were collected monthly over a 12-month period (February 2006–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 nursing piglets, finisher, and purebred boar production groups at a disproportionately 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. Upon arrival at the laboratory, fecal samples were manually stirred for 1–2 min using sterile applicator sticks to achieve fecal mixture uniformity. Four ml of feces from the 50 ml tube was transferred to a 5 ml tube that contained 1 ml of glycerol and a total of 5 replicates were produced from each original sample. The 5 ml tubes were sealed, vortexed for 1 min, and maintained at –80°C until time for cultivation of Cd.

2.3. PCR for toxin A&B genes, tcdC gene deletion, toxinotyping, and cdtB binary toxin gene

DNA extraction was accomplished by the QIamp DNA mini-kit (QIAGEN Sciences, Germantown, MD). PCR procedures for tcdA, tcdB, cdtB binary toxin, and tcdC gene detection followed the techniques according to PCR protocols as utilized by the Centers for Disease Control and Prevention (CDC), Atlanta, GA, [10–12]. Toxinotyping was performed by amplification of the toxin A and toxin B genes followed by restriction. The toxin A gene was restricted with EcoRI and the toxin B gene was restricted with Hinc II and Alcl (New England Biolabs Inc., Ipswich, MA). Restriction mixtures were left overnight in the thermocycler at 37°C and then run on a 1.5% gel to visualize the banding patterns. Patterns were compared to a toxinotype V control and toxinotype III control. Patterns differing from the two controls were compared to images in published articles [13].

2.4. Antibiotic resistance

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., Piscataway, 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 MICs for ciprofloxacin were based on values for trovafloxacin, whereas vancomycin interpretation was based on MIC’s for Gram positive aerobes. Quality control strains, Bacteroides fragilis (ATCC #25285) and B. thetaiotaomicron (ATCC #29741) were tested using recommended breakpoints for MIC’s [14].

2.5. Statistical analyses

Statistical analyses (in XTLOGIT procedure:Stata SE Release 10.1, Stata Corp., College Station, TX) were those appropriate for longitudinal 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) [15].

3. Results

3.1. Sample collection and Cd prevalence per production group

There were 1008 samples collected and tested from the following production and age groups of swine or pork: farrowing barn suckling piglets, farrowing barn lactating sows and effluent, nursery (weaned piglets), pork trim, grower/finisher pigs and

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

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>MIC S/C</th>
<th>I/R</th>
<th>Results/131 tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amoxicillin-clavulanic acid</td>
<td>≤4/2</td>
<td>8/4</td>
<td>≥16/8</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>≤0.5</td>
<td>1</td>
<td>≥2</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>≤16</td>
<td>32</td>
<td>≥64</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>≤8</td>
<td>16</td>
<td>≥32</td>
</tr>
<tr>
<td>Clindamycin</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Imipenem</td>
<td>≤4</td>
<td>8</td>
<td>≥16</td>
</tr>
<tr>
<td>Metronidazole</td>
<td>≤8</td>
<td>16</td>
<td>≥32</td>
</tr>
<tr>
<td>Piperacillin-tazobactam</td>
<td>≤32/4</td>
<td>64/4</td>
<td>≥128/4</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>≤4</td>
<td>8</td>
<td>≥16</td>
</tr>
<tr>
<td>Ciprofloxacin a</td>
<td>≤2</td>
<td>4</td>
<td>≥8</td>
</tr>
<tr>
<td>Vancomycin b</td>
<td>≤4</td>
<td>8–16</td>
<td>≥32</td>
</tr>
</tbody>
</table>

a Sensitive, Intermediate, and Resistant MIC values from CLSI [14].
b Ciprofloxacin interpretation based on MIC for trovafloxacin; vancomycin interpretation based on MIC for Gram positive aerobes.


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breeding sows/boars (Table 2). 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: 50.0\% (61/122) for sucking piglets, 23.8\% (34/143) for farrowing barn lactating sows and effluent, 8.4\% (10/119) for nursery, 6.5\% (4/62) for pork trim, 3.9\% (15/382) for grower/finisher, and 3.9\% (7/180) for breeding sows and boars.

### 3.2. PCR testing for toxin genes

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 \(cdtC\) regulatory gene, all 131 of the isolates tested positive for the \(cdtB\) 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 \(cdtC\) 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+, (not toxinotype \(V\)) isolates.

### 3.3. Seasonality/geographical location and multivariable model

The multivariable mixed model tested the following variables: production group, month, season (winter; \([\text{December, January, February}]\), spring; \([\text{March, April, May}]\), summer; \([\text{June, July, August}]\), fall; \([\text{September, October, November}]\), and geographical location. The initial unadjusted mixed model logistic regression found that the main effect for production type was significantly \((P \leq 0.001)\) associated with the probability of detecting Cd in a sample (Table 3). Month, season, and geographical location were not significantly associated with Cd presence in the initial unadjusted models \((P \geq 0.05)\). Season was not significant to the model when the four seasons were included; however, when fall and winter were collapsed into one category (September to February) and spring and summer were collapsed into a second category (March to August), they were significant \((P \leq 0.001)\). Descriptive statistics shows that 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). In the final adjusted model, interactive effects between production group and season were not significant \((P \geq 0.05)\). The intercept only model suggested that 17.4\% of the total variance was attributed to geographical location \((P \geq 0.05)\). In the final model, only 11.5\% of the total variance was attributed to location \((P \geq 0.05)\) showing that some of the variation in location was explained by the production groups at those locations.

### 3.4. Antibiotic sensitivity

CLSI MIC values and interpretative results [14] 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, piperclillin/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 [14].

### 4. Discussion

It is evident in this study that sucking piglets in the farrowing barn contributed the largest number of positive 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 [3,16,17]. The other farrowing samples (farrowing barn effluent and lactating sows) contributed the second largest number of isolates. High prevalence in this group of older animals was unexpected. The authors would have predicted the prevalence in lactating sows to be similar to that of breeding sows/boars and grower/finisher pigs. There are several reasons why sows in farrowing crates may differ from the observed pattern of declining prevalence with age. These reasons include passage of \(C. difficile\) from suckling piglets to sow, cross-contamination of feces in the crate, and true colonization of \(C. difficile\) due to stress of sow at farrowing.

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 [3,4,17,18]. In our study, 7 of the isolates were toxin A–B– and although we were unable to determine a toxinotypic, similar toxin patterns have been described for toxinotypes Xla and Xlb [19]. Two of our isolates were A-B+ and while no toxinotype was ascribed, toxin patterns would suggest that \(V\) like toxinotype could be a likely candidate [19]. The remaining 2 isolates were A+B+, but had no bp deletions in the \(tcdC\) gene. Restriction patterns were similar to those of toxinotype XXIV [19].

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

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**Table 2**

\(C. difficile\) prevalence (number positive/number tested) by production group in an integrated swine operation.

<table>
<thead>
<tr>
<th>Production Group</th>
<th>Positive/Tested (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Suckling Piglets</td>
<td>61/122 (50.0)</td>
</tr>
<tr>
<td>Other Farrow(^a)</td>
<td>34/143 (23.8)</td>
</tr>
<tr>
<td>Nursery</td>
<td>10/119 (8.4)</td>
</tr>
<tr>
<td>Pork Trim</td>
<td>4/62 (6.5)</td>
</tr>
<tr>
<td>Grow/Finish</td>
<td>15/382 (3.9)</td>
</tr>
<tr>
<td>Breeding Sows/Boars</td>
<td>7/180 (3.9)</td>
</tr>
<tr>
<td>Totals</td>
<td>131/1008 (13.2)</td>
</tr>
</tbody>
</table>

\(^a\) Includes lactating sows and effluent.

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**Table 3**

Coefficient and Odds Ratio results from the multivariable model testing the main effects and interaction of season and production groups for predicting \(C. difficile\) in swine.

<table>
<thead>
<tr>
<th>Risk factor</th>
<th>P-value (LR test d.f.)</th>
<th>Category</th>
<th>Coefficient</th>
<th>Adjusted Odds Ratio</th>
<th>OR 95% Confidence Interval</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intercept</td>
<td>–</td>
<td></td>
<td>0.31</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Season</td>
<td>&lt;0.001 (1 d.f.)</td>
<td>fall/winter (referent category)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>spring/summer</td>
<td>–0.61</td>
<td>0.55</td>
<td>0.35–0.85</td>
</tr>
<tr>
<td></td>
<td></td>
<td>sucking piglets (referent category)</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td></td>
<td>other farrow</td>
<td>–1.31</td>
<td>0.27</td>
<td>0.15–0.47</td>
</tr>
<tr>
<td></td>
<td></td>
<td>grower/finisher</td>
<td>–3.42</td>
<td>0.03</td>
<td>0.02–0.07</td>
</tr>
<tr>
<td></td>
<td></td>
<td>nursery</td>
<td>–2.55</td>
<td>0.08</td>
<td>0.04–0.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td>breeding</td>
<td>–3.36</td>
<td>0.03</td>
<td>0.01–0.08</td>
</tr>
</tbody>
</table>
months. For example, cattle shed Campylobacter spp. more heavily in the spring and autumn [20], peak human infections of Campylobacter spp. occur in mid-June [21], and ruminants have the highest fecal prevalence of E. coli O157:H7 in the summer months [22]. Somewhat in contrast to our study and although the study did not cover a 12-month period, Rodriguez-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.

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. 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 strains 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, piperacillin/tazobactam, amoxicillin/clavulanic acid, chloramphenicol, or tetracycline. Results indicate that 100% and 92% of our isolates were resistant to ciprofloxacin and clindamycin, respectively. When comparing human and animal toxino type V Cd references, it was noted that most resistance patterns overall were similar [17]. 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-B/NAP1) strains of Cd were 100% resistant to ciprofloxacin, 33% resistant to moxifloxacin and gatifloxacin, and 50% were resistant to levofloxacin and clindamycin [23]. In contrast, B/NAP1 strains were resistant to ciprofloxacin, moxifloxacin, gatifloxacin, and levofloxacin, and susceptible to clindamycin. All strains were sensitive to metronidazole and vancomycin [23].

5. Limitations, conclusions, and future directions

5.1. Limitations

Limitations to the present study would include the usual bias associated with cultivation of bacteria from environmental sources. It is unknown what the sensitivity of our culture technique was. It is obvious that Cd is difficult and time-consuming to cultivate and because of the further molecular characterization of isolates of positive samples, there are greater chances of having false negatives as opposed to false positives. Under the conditions of our sampling, we are not able to determine whether one animal or many animals contributed to a positive sample, nor were we able to quantify Cd using the methods described. The results of our antibiotic sensitivity testing may not be directly comparable to those of others because of different susceptibility testing methods, sampling techniques, and the known variable susceptibility of Cd to some antibiotics such as clindamycin.

5.2. Conclusions

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 ≤ 0.001) of Cd. The majority of our isolates were toxino type 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 ≤ 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. 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).

5.3. Future directions

We are in the process of further characterization of our isolates. Future work includes PCR ribotyping and pulsed field gel electrophoresis. 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.

Acknowledgments

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References


