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Longitudinal study comparing the dynamics of *Clostridium difficile* in conventional and antimicrobial free pigs at farm and slaughter

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ABSTRACT

Clostridium difficile is the leading cause of nosocomial diarrhea in humans and a major cause of enteritis in neonatal piglets, foals and calves. The aim of this longitudinal study was to determine and compare the prevalence, antimicrobial susceptibility, and toxinotype profiles of *C. difficile* isolated from pigs and their environment in the indoor conventional and outdoor antimicrobial free (ABF) production systems. Ten conventional and eight ABF cohorts of 35 pigs each and their environment were sampled at different stages of production at farm and slaughter. *C. difficile* prevalence in pigs was highest at the farrowing stage in both conventional (34%, 120/350) and ABF (23%, 56/244) systems, and decreased with age. This reduction in *C. difficile* prevalence in pigs at later stages of production mirrored the decreased prevalence in the farm environment. At slaughter, *C. difficile* was isolated at a low frequency from the carcasses and processing environment in both production systems. All but three isolates were resistant to ciprofloxacin (99%, 505/508), while 1.0% (5/508) and 6.0% (23/508) of isolates exhibited resistance to tetracycline and erythromycin, respectively. Toxinotype V ($tcdA^+tcdB^+$) was the predominant strain identified in both systems (conventional: 94%, 376/401; ABF: 82%, 88/107), while the rest were toxinotype XIII ($tcdA^+tcdB^+$). To conclude, we isolated antimicrobial resistant *C. difficile* regardless of antimicrobial use on the farm. Based on the phenotypic and genotypic similarity of *C. difficile* isolated in this study, we conclude that the unique production practices employed in conventional and ABF production systems have no impact on the pathogen population.

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1. Introduction

Clostridium difficile is a spore-forming anaerobic bacterium that is an important cause of enteritis in numerous food animals, including piglets, foals and calves (Baverud et al., 2003; Rodriguez-Palacios et al., 2006; Songer and Anderson, 2006). While *C. difficile* can be isolated from healthy pigs, clinical infections can result in pasty to watery diarrhea, colonic edema, and occasionally death (Songer and Anderson, 2006). In conventionally raised pigs that are given antimicrobials, *C. difficile* prevalence is typically highest in young piglets less than 10 days old and ranges from 25.9% to 100% (Alvarez-Perez et al., 2009; Hopman et al., 2011; Songer and Anderson, 2006; Thakur et al., 2010). *C. difficile* is also the leading cause of antimicrobial-associated and nosocomial diarrhea in humans in the U.S. and has recently been described as an emerging community-associated disease (CDC, 2008; McDonald et al., 2006; Noren et al., 2004). Furthermore, recent work has reported similar *C. difficile* strains in humans, various food animals and retail meat products, leading to the concern that *C. difficile* could be a zoonotic and foodborne pathogen (Debast et al., 2009; Keel et al., 2007).

Limited studies have been done in outdoor antimicrobial free pigs (Keessen et al., 2011; Nagy and Bilkei, 2003). Furthermore, no farrowing-to-slaughter study has been conducted to determine the prevalence, antimicrobial

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susceptibility, and toxinotype of *C. difficile* in outdoor antimicrobial free pigs that are not exposed to antimicrobials and compared it to the conventional system that uses antimicrobials. The main objective of this study was to compare the prevalence, antibiotic susceptibility, and toxinotypes of *C. difficile* isolated from pigs and their environment in conventional and ABF systems. Gaining a better understanding of *C. difficile* dynamics in pigs will not only allow for improvements in swine production systems but may also provide insight into potential transmission at farm and slaughter.

2. Materials and methods

2.1. Farm selection

Ten conventional and eight ABF cohorts consisting of 35 pigs each were followed and sampled throughout each stage of production (farrowing, nursery and finishing) on farm and at slaughter in North Carolina from October 2008 to December 2010. The selection of 35 pigs per cohort was based on sample size calculation using alpha (0.05) and power (0.80) coefficients. We estimated that between 27 and 35 pigs would need to be sampled to detect a statistically significant difference in the proportion of *C. difficile* positive pigs in the two production systems. Conventionally raised pigs were enrolled in sampling through contact with a large-scale commercial company (typical herd size of 700–5000 pigs/farm) with numerous hog farms in North Carolina. ABF raised pigs were enrolled through contact with individual, small-scale farmers (typical herd size of 50–200 pigs/farm) based on willingness to participate. The conventionally raised pigs sampled in this study were Yorkshire and Landrace breed pigs and followed a commercial all-in all-out (AIAO) indoor production flow. In the AIAO production system, cohorts were transported on trucks to a different farm for each production stage including the nursery and finishing stages. The pigs are housed in close quarters in pens within larger barns. Each barn was cleaned and disinfected before the next set of pigs was placed in them. The ABF raised pigs were mostly Duroc and Yorkshire breed and remained outdoors in a large pasture on the same farm until they achieved the desired market weight. However, the ABF raised pigs were rotated to a different pasture within the same farm for the nursery and finishing stages of production. Cohorts within each farm were selected based on which piglets were between 7 and 10 days old and appeared healthy. Healthy piglets were sampled to reduce loses throughout the stages of production. Pigs within the same cohort were co-mingled and housed in the same barn or pasture and each cohort was independent and from a separate farm. The conventionally raised pigs received antimicrobials for both prophylaxis and therapeutic purposes. The ABF raised pigs in this study were not given any antimicrobials at any stage of production.

2.2. Sample collection at farm and slaughter

Pig fecal samples were collected once at farrowing (7–10 days) and twice at the nursery (4 and 7 weeks) and finishing (16 and 26 weeks) stages of production. The last sample at 26 weeks was collected within 48 h of pig transport to the slaughter facility. Corresponding sow fecal samples were also collected at farrowing farms. Cohorts were ear tagged at farrowing to identify and sample the same set of pigs at each sampling point. Sample collection was attempted from all 35 pigs in the cohort at all sampling points. There were minimal losses at the different farm stages resulting in fewer than 35 fecal samples collected. Fecal samples from piglets at the farrowing stage were obtained directly from the rectum with fecal loops while samples from nursery and finishing stage pigs were collected using a clean, gloved hand.

Environmental samples were collected at each sampling point from both conventional and ABF farms. Environmental sampling consisted of five samples each of floor swabs, feed (10 g), water (10 ml), and soil (10 g) at each sampling point. Soil was collected from the exterior of the barn for conventional farms and from the pasture for ABF farms. Floor swabs were collected by wiping sterile, moist swabs five times per side on the interior of the pens for conventional farms and hoop structures, fencing and trees for ABF farms. In addition, five samples each of lagoon water (10 ml) from conventional farms were collected from the corners and next to the effluent pipes using sterile cups. Five samples each of truck floor swabs were collected from conventional systems by wiping sterile, moist swabs five times per side along the section of the truck the pigs were to be placed. ABF farms did not have lagoons or trucks.

Conventionally raised pigs were shipped on trucks to a single large-scale (10,000 pigs/day), commercial processing plant that utilized an automatic conveyer belt and processing system and a blast chiller (−30 °C) to quickly freeze the carcass. The outdoor swine producers transferred the ABF raised pigs on their own truck to one of two small-scale processing plants (250 pigs/day), in which all processes are completed manually by the staff and the carcasses are cooled overnight (1–4 °C). These small-scale slaughter facilities processed only outdoor ABF raised pigs. At slaughter, mesenteric lymph nodes (MLN), post-evisceration and post-chill swabs were collected from each pig. Post-evisceration and post-chill swabs were collected by wiping a sterile, moist swab ten times per side along the midline of the carcass from the jowl to the rear. Not all pigs within the cohort were available to sample at slaughter for numerous reasons including mortality on farm and delay in achieving the required market weight. Environmental samples at slaughter were comprised of five each of truck floor swabs and lairage swabs, where the pigs rest before they are processed. Both truck and lairage swabs were collected by wiping a sterile, moist swab five times per side on the floor of the truck or the lairage.

2.3. Bacterial isolation

C. difficile was isolated from samples by inoculating 1 g of fecal material, feed or soil and 1 ml of lagoon water into 10 ml of *C. difficile* enrichment broth (4% proteose peptone, 0.6% fructose, 0.5% sodium phosphate dibasic, 0.2% sodium chloride, 0.1% potassium dihydrogen phosphate, 0.1%

sodium taurocholate, and 0.01% magnesium sulfate, with antibiotic supplement containing cycloserine and cefoxitin (Fluka, Sigma–Aldrich, St. Louis, MI, USA)) and incubated under anaerobic conditions at 35 °C for 7 days. An ethanol shock was then performed to select for bacterial spores. The pellet was streaked onto *C. difficile* agar base (Oxoid, Cambridge, UK) with 7% laked horse blood and Fluka antibiotic supplement and incubated at 35 °C for 48 h. Presumptive colonies were tested for the production of L-prolineaminopeptidase (Pro Disc, Remel, Lenexa, KS, USA) and stored at −80 °C for further analysis. The same protocol detailed above was also used for isolating *C. difficile* from swabs and MLN samples, except different volumes were used in the initial enrichment step. For these samples, 30 ml of *C. difficile* broth was added to a single swab or a single macerated MLN and incubated under anaerobic conditions at 35 °C for 7 days. MLN samples were dipped in ethanol, flamed and cut open using sterile scissors. The DNA was extracted using the Qiagen DNeasy Blood and Tissue kit following the manufacturer's instructions (Qiagen, Hilden, Germany). PCR amplification of the housekeeping gene triose phosphate isomerase (*tpi*) was performed to confirm *C. difficile* isolates as previously described (Lemee et al., 2004).

2.4. Antimicrobial susceptibility testing

The minimum inhibitory concentration (MIC) levels of the *C. difficile* isolates were determined for six antimicrobials using E-test strips (bioMérieux, Inc., Durham, NC, USA) containing gradients of antimicrobial concentrations plated on Mueller–Hinton plates with 5% sheep blood. Antimicrobials tested with abbreviations and dilution range included ampicillin (Amp, 0.016–256 μg/ml), ciprofloxacin (Cip, 0.002–32 μg/ml), erythromycin (Ery, 0.016–256 μg/ml), metronidazole (Met, 0.016–256 μg/ml), tetracycline (Tet, 0.016–256 μg/ml), and vancomycin (Van, 0.016–256 μg/ml). Breakpoint values used in this study were based on the MIC breakpoints determined by the Clinical and Laboratory Standards Institute (CLSI) for anaerobic bacteria (CLSI, 2007). Breakpoints for antimicrobials not listed by CLSI were determined following Huang et al. (2009).

2.5. Gene identification and toxinotype identification

PCR was used to detect toxin A (*tcdA*), toxin B (*tcdB*), binary toxin (*cdtb*), and *tcdC* deletions following previously described protocols (Lemee et al., 2004; Spigaglia and Mastrantonio, 2002; Stubbs et al., 2000; Tang et al., 1994). Toxinotyping was also performed as previously described (Rupnik et al., 1998). Any atypical results were confirmed using the same PCR protocols listed above, but the DNA was extracted using another kit to obtain higher DNA yields, MasterPure Gram Positive DNA Purification Kit following the manufacturer's instructions (Epicentre, Madison, WI, USA).

2.6. Statistical analysis

Prevalence was determined at the farm level. The fecal and environmental samples collected at both the nursery and finishing stage of production were averaged to produce an average nursery and average finishing prevalence, as these samples were dependent and not significantly different. Fecal samples from the same cohort were analyzed as repeated, dependent measures. Environmental samples were collected at different farms or pastures and considered as independent measures. Significant differences in prevalence and toxinotype were estimated using general estimating equations (GEE) with an unstructured correlation structure to account for repeated measures, when appropriate, and clustering between farms, using SAS version 9.1 (SAS, Cary, NC, USA). Due to the small number of antimicrobial resistant isolates, a χ^2 test was used to calculate differences in frequency of resistance. Significance was determined at $P \leq 0.05$.

3. Results

3.1. C. difficile prevalence in pigs and farm environment

A total of 2977 conventional (1650 pig fecal, 1327 environmental) and 2035 ABF (1238 pig fecal, 797 environmental) samples were collected in this study. *C. difficile* prevalence in conventionally raised pigs was highest at farrowing (34.3%, 120/350) and decreased significantly in nursery (5.2%, 34/651) and finishing age (0.3%, 2/579) pigs ($P < 0.001$). Similar to conventional pigs, *C. difficile* in ABF raised pigs was isolated predominantly at the farrowing stage (23.0%, 56/244), with a significant decline in prevalence at the nursery stage (1.4%, 7/491) ($P = 0.001$) (Fig. 1). *C. difficile* prevalence in pigs was not significantly different between farm systems at any stage of production, with the exception of a significantly higher prevalence in conventional sows (34.3%, 24/70) than ABF sows (5.1%, 2/39) ($P = 0.009$).

C. difficile was isolated most frequently from the conventional farm environment at the farrowing stage from water (10%, 5/50), feed (16%, 8/50), soil (36.5%, 19/52) and floor swabs (66%, 33/50). However, prevalence in the lagoon samples was 8% (4/50) at farrowing, 41% (41/100) at nursery and 5.3% (5/95) at the finishing levels of production. Conventional truck floor prevalence was 62.9% (22/35) when moving conventionally raised pigs from farrowing to nursery and 31.1% (14/45) when moving pigs from nursery to finishing. Repeated sampling of the ABF farm environment also revealed higher *C. difficile* prevalence in soil (17.9%, 7/39) and swab (40%, 16/40) samples at the farrowing stage, which decreased significantly over time ($P = 0.019$, $P = 0.003$, respectively). The pathogen was isolated from a single feed sample at farrowing, but not from the water provided to the pigs at any stage of production in ABF farms. All ABF environmental samples collected at the finishing stage tested negative for *C. difficile*.

3.2. C. difficile prevalence on carcass and in slaughter environment

Pig carcass and processing environment samples collected at slaughter included 888 from conventional

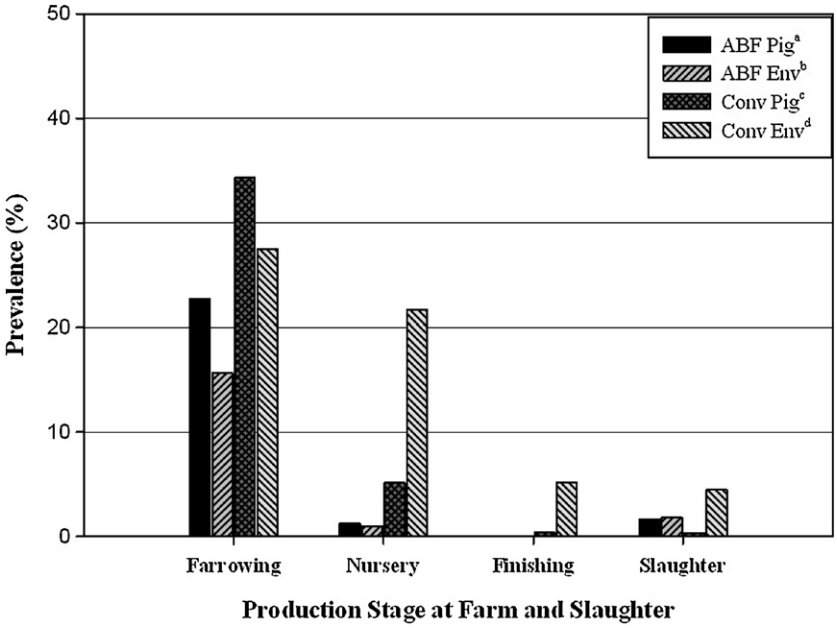

Fig. 1. *Clostridium difficile* prevalence in conventional and antibiotic free (ABF) pigs at farm, slaughter and in the environment. *X*-axis, production stage at farm and slaughter; *Y*-axis, percent prevalence of *C. difficile* in pigs and their environment at different stages on farm and slaughter. (a) Antibiotic free (ABF) pigs, sampled at different stages on farm including farrowing, nursery and finishing (sow samples were included at farrowing). (b) Environmental samples collected from ABF farm (floor swabs, feed, water and soil) and slaughter (trucks and lairage). (c) Conventionally raised pigs sampled at different stages on farm including farrowing, nursery and finishing (sow samples were included at farrowing). (d) Environmental samples collected from conventional farm (barn floor swabs, feed, water, soil, lagoon and truck floor) and slaughter (trucks and lairage).

systems (798 carcass, 90 environmental) and 680 from ABF systems (565 carcass, 115 environmental). In conventionally raised pigs, *C. difficile* was isolated from two (0.7%, 2/271) MLN samples and not from either the post-evisceration or the post-chill swabs. In ABF raised pigs, a single MLN (0.5%, 1/184), 2.2% (4/182) of post-evisceration and 2.5% (5/199) of post-chill samples tested positive for *C. difficile*. All the *C. difficile* positive post-chill carcasses originated from a single ABF farm. There were no significant differences between *C. difficile* prevalence in MLN and post-chill samples for ABF and conventional carcasses ($P = 0.696$, $P = 0.197$, respectively). Prevalence in post-evisceration samples was significantly higher for ABF (2.2%) than conventional (0%) carcasses ($P = 0.036$). It is important to note that no correlations between positive MLN, post-evisceration and post-chill samples were detected in this study. Prevalence of *C. difficile* in the conventional and ABF slaughter environment was low and not significantly different (Lairage: $P = 0.932$; Truck: $P = 0.127$). We isolated *C. difficile* from 5% (2/40) of the floors of trucks moving the conventionally raised pigs from farm to slaughter. None of the ABF producer truck floors tested positive for *C. difficile*. Four percent (2/50) of conventional and 5% (4/80) of ABF lairage samples tested positive for *C. difficile* in this study.

3.3. C. difficile antimicrobial susceptibility

Antimicrobial resistance was detected to four of six antimicrobials tested, with all the isolates exhibiting susceptibility to vancomycin and metronidazole. MIC values and frequency of resistance to different antimicrobials are highlighted in Table 1. All but three isolates were resistant to the fluoroquinolone ciprofloxacin (99.4%, 505/508). There were four antimicrobial resistance patterns identified including $Cip^{R}Ery^{R}$ (3.3%, 17/508), $Cip^{R}Ery^{R}Tet^{R}$ (1.0%, 5/508), $Cip^{R}Amp^{R}$ (1.0%, 5/508), and $Cip^{R}Ery^{R}$-Amp^{R}(0.2%, 1/508). These patterns were identified only in conventional system samples, with the exception of a single isolate with the $Cip^{R}Ery^{R}$ pattern isolated from an ABF lairage floor. Frequency of resistance to ampicillin, tetracycline and erythromycin did not differ significantly between the two production systems ($P = 0.443$, $P = 0.543$, $P = 0.081$, respectively). This is likely due to the small number of resistant isolates detected.

3.4. C. difficile gene and toxinotype profile

The majority of isolates collected in this study were $tcdA^{+}tcdB^{+}cdtB^{+}$, including 98.3% (394/401) of conventional and 96.3% (103/107) of ABF samples. The remaining isolates (2.2%, 11/508) were nontoxigenic ($tcdA^{-}tcdB^{-}$). All of the nontoxigenic isolates tested negative for the binary toxin (cdtB) and *tcdC*. No $tcdA^{-}tcdB^{+}$ isolates were detected in this study. Toxinotype V ($tcdA^{+}tcdB^{+}$) was the most commonly identified strain in both conventional (93.8%, 376/401) and ABF (81.3%, 87/107) isolates, as shown in Table 2. Toxinotype V was isolated more frequently in the conventional production system than the ABF production system ($P = 0.027$). The majority of toxinotype V isolates were binary toxin positive (98.7%, 457/463) and all carried the 39 bp deletion in *tcdC*.

Table 1
Clostridium difficile minimum inhibitory concentration (MIC) levels and frequency of resistance at farm and slaughter in the two production systems.

Antimicrobials[a]	Pig		Farm environment		Carcass		Slaughter environment	
	ABF (n = 65)	Conv (n = 180)	ABF (n = 28)	Conv (n = 215)	ABF (n = 10)	Conv (n = 2)	ABF (n = 4)	Conv (n = 4)
Tetracycline								
MIC_{50} [b]	2.50	3	0.05	3	0.09	6.01	2	2
MIC_{90} [c]	4	6	4.80	8	3.30	10.80	2.70	2
%*R* [d]	0	0	0	2.30	0	0	0	0
Ampicillin								
MIC_{50}	0.75	0.50	0.75	0.50	1	0.55	0.63	0.22
MIC_{90}	1.50	1	1	1	1.50	0.91	0.93	0.34
%*R*	0	1.70	0	1.40	0	0	0	0
Metronidazole								
MIC_{50}	0.19	0.19	0.19	0.19	0.38	0.11	1.56	0.19
MIC_{90}	0.38	0.75	0.25	1.75	6.20	0.12	5.10	0.23
%*R*	0	0	0	0	0	0	0	0
Erythromycin								
MIC_{50}	0.75	0.50	0.75	0.50	0.88	0.88	0.63	0.32
MIC_{90}	1	1	1	1	1	1.38	179.43	0.38
%*R*	0	5	0	6	0	0	25	0
Ciprofloxacin								
MIC_{50}	>32	>32	>32	>32	>32	>32	>32	>32
MIC_{90}	>32	>32	>32	>32	>32	>32	>32	>32
%*R*	98.5	99.4	100	99.5	100	100	100	100
Vancomycin								
MIC_{50}	0.50	0.38	0.75	0.38	1	0.50	0.50	0.38
MIC_{90}	1	0.52	0.85	0.75	1	0.50	0.50	0.46
%*R*	0	0	0	0	0	0	0	0

[a] Antimicrobial breakpoint: tetracycline, ≥16; ampicillin, ≥2; metronidazole, ≥32; erythromycin, ≥8; ciprofloxacin, ≥8; vancomycin, ≥32.
[b] Minimum inhibitory concentration of the antimicrobial that inhibits the growth of 50% of the isolates tested.
[c] Minimum inhibitory concentration of the antimicrobial that inhibits the growth of 90% of the isolates tested.
[d] Percent resistant isolates.

Toxinotype XIII ($tcdA^+tcdB^+$) was identified in 4.5% (18/401) of conventional and 15.0% (16/107) of ABF isolates. All toxinotype XIII isolates were binary toxin negative and carried the 18 bp deletion in *tcdC*. Additionally, 64.7% (22/34) of all toxinotype XIII were isolated in the nursery stage and 41.2% (14/34) exhibited resistance to erythromycin. Seven out of the eight cohorts with toxinotype XIII isolates were detected at only one sampling point (most commonly the nursery stage). There was a single conventional cohort with toxinotype XIII isolates at both the finishing stage and in the MLN at slaughter. Toxinotype XIII was detected in both pig and farm environmental samples in three of the four ABF and one of the four conventional cohorts. On these farms, toxinotype XIII was only isolated in soil, swab or lagoon samples. It is also interesting to note that while toxinotype XIII was often isolated with toxinotype V, there were two sets of samples, one finishing stage conventional and one nursery stage ABF, where toxinotype XIII was the only strain detected.

4. Discussion

The objective of this longitudinal study was to determine the prevalence, antimicrobial susceptibility,

Table 2
Toxin genes and toxinotype of *Clostridium difficile* isolated in the two production systems.

Production system	Toxinotype V (A^+B^+)	Toxinotype XIII (A^+B^+)	Nontoxigenic (A^-B^-)
Conventional			
Pig (n = 180)	94.4% (170)[a]	5.0% (9)	0.6% (1)
Farm environment (n = 215)	93.5% (201)	3.7% (8)	2.8% (6)
Carcass (n = 2)	50.0% (1)	50.0% (1)	0%
Slaughter environment (n = 4)	100.0% (4)	0%	0%
ABF			
Pig (n = 65)	78.5% (51)	16.9% (11)	4.6% (3)
Farm environment (n = 28)	85.7% (24)	14.3% (4)	0%
Carcass (n = 10)	90.0% (9)	0%	10.0% (1)
Slaughter environment (n = 4)	75.0% (3)	25.0% (1)	0%

[a] Indicates percent (number).

and toxinotype of *C. difficile* in conventionally and ABF raised pigs and their environment, throughout stages of production and at slaughter. To the author's knowledge, this is the first longitudinal study to report *C. difficile* dynamics in the ABF swine production system. Prevalence was highest at the farrowing stage, including the conventionally raised piglets (34%, 120/350), ABF raised piglets (23%, 56/244), and the majority of environmental samples, as shown in Fig. 1. The significant reduction in prevalence at the nursery and finishing stages of production is consistent with other reports (Hopman et al., 2011; Thakur et al., 2010; Yaeger et al., 2002). One potential reason explaining this decline is the role played by the development of a strong immune response and out competition by other commensal bacteria in the pig gut (Bergogne-Berezin, 2000; Kyne et al., 2001). Conventional sow prevalence in this study was similar to previous reports (Thakur et al., 2010; Weese et al., 2010) and significantly higher than ABF sow prevalence ($P = 0.009$). The reason for the difference in sow prevalence between the two production systems remains unclear. Potential factors contributing to a lower prevalence in ABF sows could be the surrounding environment, diet, pig breed, stress or immunity (Kyne et al., 2001; Nagy and Bilkei, 2003; Yaeger et al., 2002).

It is interesting to highlight that the prevalence of *C. difficile* in the conventional indoor and ABF outdoor environment reciprocated with the pig level prevalence. The interior of the conventional barn is cleaned before the next set of pigs is place in them while the ABF piglets are moved into a different pasture for later stages of production. This could be an important factor in the decline in prevalence found at nursery and finishing stages. Floor and ground swab prevalence in the individual conventional barns and ABF outdoor farm environment was higher than the corresponding piglet and sow prevalence in both production systems at farrowing. This finding indicates the farrowing environment as a potential reservoir of *C. difficile* for piglets in both production systems. In the future, extensive sampling and testing of the farrowing environment may lead better control measures, thus reducing the burden of *C. difficile* in farrowing farms. Conventional truck samples were also higher than corresponding fecal samples at both the nursery and finishing levels, indicating a potential reservoir for acquisition of *C. difficile*.

Clostridium difficile was isolated at a low frequency at slaughter in both conventional and ABF raised pigs. The higher prevalence in post-evisceration samples from ABF raised pigs compared to conventionally raised pigs may be the result of the different processing methods used by the slaughter plants discussed earlier. To the author's knowledge, this is the first study to examine prevalence of *C. difficile* in the mesenteric lymph nodes of pigs intended for food production at slaughter. The positive MLN samples in both production systems originated from farms that tested negative for *C. difficile* at the finishing stage of production. This clearly indicates that the pathogen does exist in the MLN and could serve as a site for contaminating carcass at processing. *C. difficile* has been shown to cross the intestinal mucosa and translocate to the MLN in gnotobiotic mice (Debure et al., 1987).

The positive post-chill samples are important as they closely represent the final retail product and are the most important for public health. It is also important to note that these positive post-chill samples originated from a single ABF farm, which tested negative for *C. difficile* in all the pigs, farm environment and slaughter environment samples. While it cannot be confirmed, it is plausible the *C. difficile* isolated on the post-chill carcasses originated from inside the processing facility. The detection of *C. difficile* on ABF post-chill carcasses and not conventional carcasses could be the result of different processes used to cool the carcass. Additional testing of post-chill carcasses and the processing environment is important to prevent contaminated carcasses from reaching consumers.

Antimicrobial resistance to the fluoroquinolone ciprofloxacin was ubiquitous among the *C. difficile* isolates in this study (99%, 505/508), regardless of whether the animal was exposed to antimicrobials on the farm. This is in agreement with other studies that have reported high prevalence of ciprofloxacin resistant *C. difficile* in conventionally raised pigs as well as humans without previous exposure (Gerding, 2004; Norman et al., 2009). This finding is particularly surprising in ABF raised pigs because they are not given any antimicrobials at any stage of their production. The dominance of *C. difficile* strains resistant to ciprofloxacin in both production systems may be the result of these strains being more ecologically fit than ciprofloxacin-susceptible strains. This has been demonstrated in *Campylobacter*, where fluoroquinolone-resistant strains are better fit to survive than susceptible strains, even in the absence of any antimicrobial selection pressure (Zhang et al., 2006). Resistance to the two major drugs of choice in humans, metronidazole and vancomycin, was not detected.

The vast majority of isolates in this study were $tcdA^{+}tcdB^{+}$. Additionally, most were binary toxin positive and carried the 39 bp mutation in the toxin regulatory gene, *tcdC*. Toxinotype V and XIII were the only toxinotypes detected in this study, with toxinotype V representing a majority of both fecal and environmental isolates in both production systems, as illustrated in Table 2. This is in agreement with other studies that have found toxinotype V to be a common strain detected in pigs at farm and slaughter (Baker et al., 2010; Debast et al., 2009). It appears that *C. difficile* toxinotype V has created a suitable niche for itself in both the conventional and ABF swine production system. In agreement with previous reports, the vast majority of toxinotype V isolates were also binary toxin positive, carried the 39 bp deletion in *tcdC*, and were resistant to the fluoroquinolone ciprofloxacin (Jhung et al., 2008; Norman et al., 2009). While the detection of binary toxin negative toxinotype V isolates is surprising, it has been previously reported (Gonçalves et al., 2004; Jhung et al., 2008).

A markable find was the detection of toxinotype XIII in both production systems, including four conventional and four ABF cohorts. Toxinotype XIII has not been widely reported in the literature, and this is the first report indicating its presence in pigs. Mutlu et al. (2007) identified a single toxinotype XIII isolate from a hospital in Scotland, which was characterized as ribotype 001 and

erythromycin resistant. It is interesting to note that 42%(14/34) of toxinotype XIII isolates detected in this study were also resistant to erythromycin. The isolation of toxinotype XIII isolates primarily in a single production stage is also interesting. This could indicate that pigs can transiently pick up a different strain in a new environment, even when toxinotype V is the main strain detected in that environment.

5. Conclusion

The results of this study highlight the similarity in prevalence, antimicrobial susceptibility and toxinotype of *C. difficile* isolated from both pig and environmental samples in conventional and ABF production systems in North Carolina, despite the significant differences in production practices, including indoor or outdoor production and antibiotic use. These results suggest that, unlike human cases of *C. difficile*, the use or absence of antibiotics does not affect the prevalence of *C. difficile* in pigs. The high prevalence of toxinotype V isolates indicates this strain is well adapted to pigs and their environment, regardless of the indoor or outdoor production system. While not isolated as frequently, this study is the first to identify toxinotype XIII in conventionally and ABF raised pigs.

Ethics

Protocols involving pigs were approved by the Institutional Animal Care and Use Committee at North Carolina State University.

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