Prevalence of Clostridium difficile in retail pork

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Abstract — Clostridium difficile was isolated from 1.8% (7/393) of retail pork samples obtained from 4 Canadian provinces. Five ribotypes and 3 toxinotypes were identified. Three isolates were indistinguishable from the international outbreak strain ribotype 027 and were toxinotype III. Although the implications for food safety practices remain elusive, the frequency of toxigenic isolates and isolates indistinguishable from known human pathogenic strains suggests contaminated pork may be a source of C. difficile in humans.

Résumé — Prévalence de Clostridium difficile dans le porc au détail. Clostridium difficile a été isolé chez 1.8 % (7/393) des échantillons de porc de détail obtenus dans 4 provinces canadiennes. Cinq ribotypes et 3 toxinotypes ont été identifiés. Trois isolats étaient indistinguables de la souche d’éclosion internationale ribotype 027 et étaient de toxinotype III. Même si les répercussions pour la salubrité des aliments demeurent difficiles à définir, la fréquence des isolats toxicogéniques et des isolats indistingubles des souches pathogènes humaines connues suggère que le porc contaminé peut être une source de C. difficile chez les humains.

Introduction

Clostridium difficile is an important, anaerobic, spore-forming pathogen associated with diarrheal diseases in multiple species including humans, dogs, horses, and pigs (1–5). It is of growing concern to the human healthcare community due to the increase in the frequency and severity of C. difficile infection (CDI) (6,7). This increase has been attributed, at least in part, to the emergence of a highly virulent strain designated ribotype 027, North American pulsotype1 (NAP1) and restriction endonuclease type B1 (8). This strain produces high levels of toxins A and B in vitro (9), likely because of a truncating mutation (Δ117) in the tcdC gene, a negative regulator of toxins A and B production (2). There have also been reports suggesting that CDI may be an overlooked but increasing cause of disease in humans in the community (10). Recent studies have suggested that ribotype 078, a toxinotype V strain, may be associated with community-associated disease.

This strain also has an alteration in the tcdC gene, a nonsense mutation at position 185 (C184T) (11).

Clostridium difficile can be isolated from varying percentages of healthy animals, including food-producing animals such as calves (12), pigs (4), and chickens (13). Further, strains found in food animals are often those implicated in CDI (11,12). Of particular concern is the isolation of ribotypes 027 and 078 from meat (12). Because of the presence of important strains of C. difficile in food animals, concern has been expressed about the potential for foodborne transmission (14). To date, few studies assessing C. difficile contamination of food products have been performed.

Rodriguez-Palacios et al (15) found C. difficile contamination in 20% of Canadian retail ground beef they sampled. Songer et al (16) reported high levels of contamination in retail beef, pork, and turkey products (44%, 47%, 38%, respectively), while an earlier study isolated C. difficile from approximately 3% of raw vegetables in the UK (17).

In general, these studies used relatively small sample sizes and/or limited geographic ranges. Furthermore, clustering of data may have occurred in these studies, potentially biasing the findings. A better understanding of C. difficile contamination of food products is required to help evaluate the risks of food in CDI and develop appropriate prevention and control mechanisms, if required. The objective of this study was to determine the prevalence of C. difficile contamination in retail pork from across Canada.

Materials and methods

Sample collection and processing

Between November 2007 and May 2008, 393 retail pork samples (ground pork and pork chops) were collected from...
4 provinces: British Columbia, Saskatchewan, Ontario, and Quebec. Single samples were purchased from retail outlets using the systematic sampling method used for the retail component of the Canadian Integrated Program for Antimicrobial Resistance Surveillance (CIPARS) (18). Samples were shipped to the study laboratory on ice. Samples that weren’t processed immediately were either refrigerated for up to 4 d or frozen at −80°C.

Sections of pork (~15 g each) were placed in sterile containers and covered with 50 mL of *Clostridium difficile* growth medium consisting of 40 g/L proteose peptone, 5.0 g/L disodium hydrogen phosphate, 1.0 g/L potassium dihydrogen phosphate, 0.1 g/L magnesium sulfate, 2.0 g/L sodium chloride, 6.0 g/L fructose, and 0.1% (wt/vol) sodium taurocholate. Samples were incubated anaerobically at 37°C. Cultures were alcohol shocked for spore selection after 7 d incubation; then 2 mL of the cultures was mixed with 2 mL of ethanol, vortexed briefly, and incubated at room for 1 h. Cultures were then centrifuged at 4000 × g for 10 min at 4°C. The pellet was plated onto Columbia Blood agar (CAB) (Oxoid, Nepean, Ontario) and incubated anaerobically at 37°C for 48 h. Five probable *C. difficile* colonies, as determined by colony morphology and characteristic odor were subcultured onto CAB plates and incubated anaerobically for another 48 h. Identification of *C. difficile* was confirmed based on a positive l-proline aminopeptidase activity test (Prodisk; Remel, Lenexa, Kansas, USA) and amplification of the triose phosphate gene fragment (19).

**Isolate characterization**

Detection of tcdA (toxin A), tcdB (toxin B), cdtB (binary toxin) and ribotyping were all performed as previously described (19–21). For ribotyping, gels were read visually. In situations where the ribotype was known to be a recognized international ribotype from the PHLS Anaerobic Reference Unit by previous typing of reference strains, the appropriate numerical designation (ribotype 027) was used. In other situations, internal designations (letters) were used. The tcdC gene was amplified and sequenced using the C1 (5′ TTA ATT AAT TTT CTC TAC AGC TAT CC 3′) and C2 (5′ TCT AAT AAA AGG GAG ATT GTA TTA TG 3′) primers described by Spigaglia and Mastrantonio (22). Toxinotyping was adapted from Rupnik et al (23). Briefly, the A3 and B1 PaLoc fragments were amplified by polymerase chain reaction (PCR) using the following conditions: 3 min denaturation step at 93°C, 30 cycles for the A3 fragment, and 35 cycles for the B1 fragment of 8 min at 47°C followed by 3 s at 93°C, and a final extension for 10 min at 47°C. Each PCR contained 1× PCR buffer, 3 mM MgCl₂, 800 μM dNTPs, 40 pmoles of each primer, 0.25 U of *taq* polymerase; 100 μM TMA was included in the A3 PCR. Antimicrobial resistance testing was performed using E-tests (AB Biodisk, Solna, Sweden) to determine minimum inhibitory concentrations (MICs) against 4 antimicrobials, namely, levofloxacin, metronidazole, vancomycin, and clindamycin. Breakpoints for both clindamycin and levofloxacin were ≤ 2 μg/mL (susceptible), 4 μg/mL (intermediate), and ≥ 8 μg/mL (resistant); breakpoints for metronidazole were ≤ 8 μg/mL (susceptible), 16 μg/mL (intermediate), and ≥ 16 μg/mL (resistant) (24). There are no established Clinical and Laboratory Standards Institute (CLSI) breakpoints for vancomycin, so the same breakpoints were used as previously described (25).

**Results and discussion**

Due to low background levels of *C. difficile* in the meat samples, the use of a non-selective culture medium was deemed appropriate and easily facilitated the identification of *C. difficile* from samples. *Clostridium difficile* was isolated from 7 (1.8%) of 393 retail pork samples collected from 4 provinces over a 7-month period (Table 1), including 2/71 (2.8%) samples from British Columbia, 3/93 (3.2%) samples from Ontario, 2/102 (1.96%) samples from Quebec, and 0/127 samples from Saskatchewan. There was no statistically significant association between prevalence of *C. difficile* and province (P = 0.28).

The 7 isolates were classified as 5 ribotypes (Table 1). Two isolates from Ontario and 1 from Quebec shared an identical ribotype profile with the ribotype 027 epidemic strain (Table 1). All 3 isolates had genes for toxin A, toxin B, the binary toxin, and were toxinotype III. In addition, they all had tcdC genes with the Δ117A truncating mutation (26). One other isolate from Quebec (ribotype Y) was also toxinotype III, possessed genes for all toxins, and had a mutation in the tcdC gene, but had a ribotype pattern distinct from ribotype 027. One isolate from Ontario (ribotype V) had both toxin A and B genes; however, it lacked the genes for the binary toxin. It had no significant mutations in tcdC and was determined to be toxinotype XXVI. One isolate from British Columbia (ribotype T) lacked the binary toxin genes but was positive for both toxin A and B genes. It was determined to be toxinotype 0 and had no deletions in the tcdC gene. The other isolate from British Columbia had no toxin genes and was therefore not typeable by toxinotyping. It was classified as ribotype OVC B.

The most common ribotype identified from the pork samples was the 027 human epidemic ribotype. This has been previously reported and suggests a possible human health concern. This strain has been associated with outbreaks of CDI internationally.
(27) and there is evidence that it may be highly virulent (9). This has been attributed to the presence of an alteration in the tcdC gene, which is a negative regulator of toxin A and B production. This alteration was confirmed in 027 isolates from pork in this study. Additionally, 1 other isolate possessed similar characteristics to 027, including the Δ117 mutation in tcdC. It is reasonable to expect that this strain would have similar hypervirulence properties. Ribotype 027 or related strains have been identified in retail beef (15) and pork (16). In addition to ribotype 027, ribotypes Y and T have been identified in humans with CDI in Ontario (25). A strain with an indistinguishable ribotype pattern compared to ribotype V has been found in humans with CDI in the province, yet it was of a different toxinotype and the relationship between the 2 strains is unclear. The only isolate with a ribotype not previously found in humans is OVC B, a non-toxigenic strain. Of note, ribotype 078 or toxinotype V strains, the most common isolates from pigs in some regions (4,28), were not isolated from these samples. Songer et al (16) reported 68.4% of American retail meat isolates, including beef, pork, and turkey, were ribotype 078. The finding of ribotype 078 or related toxinotype V strains in meat has raised concerns because of the recent association between these strains and community-associated CDI in humans. The absence of this type from the Canadian samples suggests 078 may generally not be as prevalent in Canada as in the United States; however, a prospective study of C. difficile colonization of pigs in Canada is required. Songer et al (16) did report the second most common ribotype from beef and pork samples to be 027 (31.4%), which approximates the proportion of ribotype 027 isolates found in this study (42.9%).

All isolates tested were susceptible to both metronidazole and vancomycin. One isolate, ribotype Y, was resistant to clindamycin and all other isolates were considered intermediate. The OVC B, and all 3027 isolates were resistant to levofloxacin. The ribotype T isolate was susceptible and the remaining isolates were intermediate. Resistance to clindamycin and levofloxacin is becoming increasingly common in human C. difficile isolates. Martin et al (25) reported that 98.4% of isolates were fully resistant to levofloxacin and the remaining 1.6% were intermediate; therefore, the identification of a susceptible isolate from pork was unexpected. As high as 92% of recent human ribotypes 027 isolates were reportedly clindamycin resistant (25) but only 1 isolate from this study was fully resistant.

The prevalence of C. difficile in pork in this study is remarkably lower than prevalences reported by Songer et al (16) in pork (44%) or Rodriguez-Palacios et al (15) in beef (20%). This may be due to the large sample size used herein and the broad geographical area from which sampling was performed. In a subsequent study by Rodriguez-Palacios et al (29), meat samples from a broader geographical range were cultured and only 6.1% had C. difficile contamination. Factors, such as regional and species differences in C. difficile colonization, as well as slaughtering and meat handling factors may also have influenced the prevalence. Further study of the sources of C. difficile contamination in meat is required.

Although standard selection and isolation procedures were performed in this study, there is a need for validated methodology for the culture, selection, and isolation of C. difficile from meat samples. The effect of different culture media or isolation procedures on C. difficile isolation from meat is not known. There is also a need for broader prevalence studies to identify other potential sources of C. difficile and to assess the contribution to disease.

This study is the first report of the prevalence of C. difficile in Canadian retail pork. The study demonstrated that C. difficile strains implicated in CDI in humans, while uncommon, were found in retail pork. Since humans and animals tend to harbor strains of the same ribotype, it is difficult to determine the source of the contamination. These isolates may have been harbored by the animal or introduced during processing or packaging post-slaughter. Although the actual risk of foodborne exposure to C. difficile spores remains unknown, spore formers, such as C. difficile, offer a unique challenge to the food industry as spores are resistant to many disinfectants and cooking temperatures and it can be difficult to eliminate these organisms from food products. Despite the low prevalence of C. difficile in pork, the role of food in CDI and the implications for food safety practices warrant further research.

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References


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