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# Detection and Enumeration of *Clostridium difficile* Spores in Retail Beef and Pork▽

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**Recent studies have identified *Clostridium difficile* in food animals and retail meat, and concern has been raised about the potential for food to act as a source of *C. difficile* infection in humans. Previous studies of retail meat have relied on enrichment culture alone, thereby preventing any assessment of the level of contamination in meat. This study evaluated the prevalence of *C. difficile* contamination of retail ground beef and ground pork in Canada. Ground beef and ground pork were purchased from retail outlets in four Canadian provinces. Quantitative and enrichment culture was performed. *Clostridium difficile* was isolated from 28/230 (12%) samples overall: 14/115 (12%) ground beef samples and 14/115 (12%) ground pork samples ($P = 1.0$). For ground beef, 10/14 samples (71%) were positive by enrichment culture only. Of the 4 ground beef samples that were positive by direct culture, 20 spores/g were present in 2 while 120 and 240 spores/g were present in 1 each. For ground pork, 10/14 (71%) samples were positive by enrichment culture only. Of the 4 ground pork samples that were positive by direct culture, 20 spores/g were present in 3 while 60 spores/g were present in 1. Ribotype 078 predominated, consistent with some previous studies of *C. difficile* in food animals. Ribotype 027/North American pulsotype 1 was also identified in both retail beef and pork. This study has identified relatively common contamination of retail ground beef and pork with *C. difficile* spores; however, the levels of contamination were very low.**

*Clostridium difficile* is an important cause of enteric disease in humans. It is the most commonly diagnosed cause of hospital- and antimicrobial agent-associated diarrhea in people, and recent evidence suggests that it may be emerging as an important community-associated pathogen (2, 5). In addition to humans, *C. difficile* can be found in the intestinal tracts of a variety of animal species, including food animals, such as cattle and pigs (7, 10, 13). *Clostridium difficile* has also been found in retail meat (11, 12, 17), and concerns about the role of food in the epidemiology of community-associated *C. difficile* infection (CA-CDI) have been expressed (5, 8, 15).

Initial studies have reported isolation of *C. difficile* from 4.6 to 45% of retail meat samples (11, 12, 17). However, all studies have used broth enrichment protocols, which could detect very low spore numbers and provide no information about the number of organisms present in a sample. No studies have evaluated numbers of *C. difficile* spores in food. While the infectious dose is not known, an understanding of the level of contamination may be an important factor in determining the relevance of contamination of food. Additionally, the use of different methods between studies hampers comparison of results. Recently a study was performed to evaluate different methods for qualitative and quantitative detection of *C. difficile* (21). This study determined that the detection threshold of enrichment culture could be at least as low as 10 spores/g of meat. It also determined that quantitative culture can accurately determine the level of contamination in experimentally inoculated meat samples, albeit with a higher detection threshold. The objective of this study was to determine the prevalence of *C. difficile* contamination of retail ground beef and ground pork using both qualitative and quantitative methods.

## MATERIALS AND METHODS

Ground beef and ground pork were purchased from retail outlets between August 2008 and November 2008 in four Canadian provinces (British Columbia, Saskatchewan, Ontario, and Quebec) as part of the Canadian Integrated Program for Antimicrobial Resistance Surveillance. Retail sampling is weighted by population. Using Statistics Canada data, between 17 census divisions are randomly selected per province by stratified random selection. The strata are formed by the cumulative population quartiles from a list of divisions in a province sorted by population in ascending order, and stores within those divisions are sampled. Field workers in Ontario and Quebec conduct one sampling day per week, while biweekly sampling is performed in the other two provinces. In each census division, four stores are selected prior to the sampling day based on store type. Three chain stores and one independent market or butcher shop are selected, except in densely population regions, where two chain stores and two independent markets or butcher shops are sampled to reflect the shopping behavior of the subpopulation. From each store, one sample of each commodity is obtained. Where possible, stores are sampled only once per year. Samples are purchased and shipped via 24-h courier in their original packages, on ice, to the study laboratory and processed within 72 h of receipt.

Qualitative testing was performed using broth enrichment. A rinse was performed by adding 25 g of ground meat to 25 ml of phosphate-buffered saline (PBS) (pH 7.4) in a sterile container. After thorough mixing by hand, 1 ml of PBS-and-meat mixture was removed and inoculated into 9 ml of *C. difficile* moxalactam (latamoxef)-norfloxacin (CDMN) broth with 0.1% sodium taurocholate and incubated anaerobically at 37°C for 48 h. An aliquot of the broth was alcohol shocked with an equal amount of anhydrous ethanol for 1 h. This mixture was then centrifuged for 10 min at 4,000 rpm. The supernatant was discarded, and the pellet was streaked onto a CDMN agar plate and incubated anaerobically at 37°C for 48 h. Suspicious colonies were subcultured on blood agar and confirmed as *C. difficile* by Gram stain appearance, colony morphology, characteristic odor, and production of L-proline aminopeptidase.

Quantitative testing was performed on the rinse solutions, prepared as described above. Serial 10-fold dilutions were performed in PBS (pH 7.4), and 100-μl aliquots were inoculated on CDMN agar. Plates were incubated, and *C. difficile* was identified as described above.

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TABLE 1. *Clostridium difficile* contamination of retail ground beef and ground pork samples from four Canadian provinces

| Province | No. (%) of positive samples | | |
|---|---|---|---|
| | Ground beef | Ground pork | Overall |
| A | 1/16 (6.3) | 0/14 (0) | 1/30 (3.3) |
| B | 3/40 (7.5) | 6/35 (17) | 9/75 (12) |
| C | 6/40 (15) | 7/57 (12) | 13/97 (13) |
| D | 4/19 (21) | 1/9 (12) | 5/28 (18) |

Isolates were typed by PCR ribotyping as has been described elsewhere (3). In situations where the ribotype was known to be a recognized international ribotype from the PHLS Anaerobic Reference Unit (Cardiff, United Kingdom) by previous typing of reference strains, the appropriate numerical designation (e.g., 027) was used. Otherwise, a letter that corresponded to an internal nomenclature for types not validated with PHLS reference strains was used. Genes encoding production of toxins A (*tcdA*) and B (*tcdB*) were evaluated using PCR (6, 9). Detection of CDT (binary toxin) was performed using PCR directed at *cdtB*, the binding component (19). Toxinotyping was performed on a representative of each ribotype (16). Additionally, the *tcdC* gene from a representative of each ribotype was amplified and sequenced as described previously (18).

Fisher's exact test or chi-square tests were used for categorical comparisons. Agreement between direct and enrichment culture was determined using the kappa statistic.

## RESULTS

*Clostridium difficile* was isolated from 28/230 (12%) samples overall: 14/115 (12%) ground beef samples and 14/115 (12%) ground pork samples ($P = 1.0$). For ground beef, 10/14 (71%) were positive by enrichment culture only while 2/14 (14%) were positive by both enrichment and direct culture and 2/14 (14%) were positive by direct culture only. Of the four ground beef samples that were positive by direct culture, 20 spores/g were present in two while 120 and 240 spores/g were present in one each.

For ground pork, 10/14 (71%) samples were positive by enrichment culture only while 2/14 (14%) were positive y both enrichment and direct culture and 2/14 (14%) were positive by direct culture only. Of the four ground pork samples that were positive by direct culture, 20 spores/g were present in three while 60 spores/g were present in one. For both ground beef and ground pork, all samples that were positive by direct but not enrichment culture contained only 20 spores/g. Agreement between direct and enrichment culture was poor (kappa = 0.22).

There was no statistically significant difference in the prevalence of contamination of meat from different provinces, either overall ($P = 0.38$) or for ground beef ($P = 0.42$) or ground pork ($P = 0.43$) individually (Table 1).

Typing data are presented in Table 2. All ribotype 078 isolates had a 39-bp deletion in the *tcdC* gene and a nonsense mutation at position 184 (C184T). All ribotype 027 and C isolates had an 18-bp deletion in *tcdC* and a single nucleotide deletion at position 117, which introduces a frameshift mutation. The other isolate, the only one not possessing CDT genes, had a normal *tcdC* gene.

TABLE 2. Molecular characteristics of *Clostridium difficile* isolates recovered from retail pork and beef

| Product | Ribotype | Toxinotype | Presence of toxin genes | No. (%) of samples |
|---|---|---|---|---|
| Pork | 078 | V | $A^+ B^+ CDT^+$ | 10 (71) |
| | 027 | III | $A^+ B^+ CDT^+$ | 1 (7.1) |
| | C | IX | $A^+ B^+ CDT^+$ | 1 (7.1) |
| | E | 0 | $A^+ B^+ CDT^-$ | 1 (7.1) |
| | Y | III | $A^+ B^+ CDT^+$ | 1 (7.1) |
| Beef | 078 | V | $A^+ B^+ CDT^+$ | 12 (86) |
| | 027 | III | $A^+ B^+ CDT^+$ | 1 (7.1) |
| | C | IX | $A^+ B^+ CDT^+$ | 1 (7.1) |

## DISCUSSION

This is the first study to quantify *C. difficile* contamination in retail meat, and the finding of low levels of contamination may be important. While the infectious dose for *C. difficile* is not known and presumably varies greatly between healthy individuals and those with underlying risk factors (i.e., antimicrobial use, comorbidities, or chemotherapeutic treatment), it is plausible that low numbers of spores are less relevant than larger numbers. The enrichment method that was used in this study has a detection threshold of $\leq$10 spores/g (21), while the detection threshold of the quantitative procedure is somewhat higher (20 spores/g). Since most samples that were positive were positive only by enrichment culture, it can be assumed that low levels of *C. difficile* are typically present in contaminated meat samples. However, in the absence of information about infectious dose and risk factors for CA-CDI, this low-level contamination should not be dismissed. The highest level of contamination that was detected was only 240 spores/g, which is quite low.

The prevalence of *C. difficile* found in this study is similar to that found in other recent Canadian studies (11, 12) but is lower than that reported in a study from the United States (17). This should not be taken as an indication of regional variation in contamination because of the different methods that were used and the different products that were tested. Standardization of sampling and culture methodology is required to evaluate interregion variation in prevalence.

It was interesting that *C. difficile* was isolated using direct but not enrichment culture in a small number of samples. The enrichment method is more sensitive than direct culture (21), so this result may seem counterintuitive. However, this could be explained by nonhomogenous distribution of *C. difficile* in meat samples, particularly with the low level of contamination that was present in most samples. All four samples that were positive by direct culture but negative by enrichment contained only 20 spores/g, perhaps giving support to the hypothesis that these samples had low-level and nonhomogenous distribution; however, that cannot be proven. The potential for nonhomogenous distribution of spores could have an impact on prevalence studies, since an underestimation of the true prevalence could be obtained. Using larger volumes of meat could be one way to reduce the potential impact of spore distribution, but this requires further study. Further study of culture methods is also required. While the methods used here were evaluated in comparison with a few other methods (21), broader investigation of various techniques is required. A better understanding of the source of contamination of meat might also provide information to guide selection of culture techniques. For ex-

ample, if *C. difficile* spores reside within muscle tissue, as has been reported for other clostridia in equine muscle (20), rinse methods such as that used here could underestimate the prevalence.

Much of the concern about the role of animals in CA-CDI has revolved around the finding of important human strains in food animals, particularly ribotype 078/toxinotype V and ribotype 027/toxinotype III (5, 8, 15). One earlier study of retail meat reported that 73% of isolates were ribotype 078 with 27% being ribotype 027 (17). Another retail meat study did not find either ribotype 027 or 078, but the most common strain was closely related to ribotype 027, being toxinotype III and classified as North American pulsotype 1, the PFGE designation for ribotype 027 (12). In this study, ribotype 078 predominated while ribotype 027 and a related toxinotype III strain were also found. These obviously raise concerns because of the role of ribotype 027 in human disease and recent evidence that ribotype 078 may be overrepresented in CA-CDI (5). Food exposure is one logical area to investigate when considering the emergence of ribotype 078 in CA-CDI. While concurrent identification of this strain in food and CA-CDI cases certainly cannot be assumed to be a causal link, it indicates a need to continue to investigate potential risks associated with food.

The typing data are also interesting in comparison to previous reports involving food animals. The predominance of ribotype 078 is consistent with some previous reports of *C. difficile* in food animals (4, 7) but curiously is in contrast to previous Canadian studies where this strain was rarely identified in food animals (13, 14). The reason for this is unclear. It is possible that the low prevalence of 078 we have previously reported was not representative of the situation across Canada or that ribotype 078 is an emerging strain in Canadian food animals and was therefore rare in earlier studies. Broader testing of food animals in different regions of Canada is required to further evaluate these possible reasons.

Much more work is required to determine whether *C. difficile* contamination of retail meat is of clinical relevance, and results should be taken in context with studies that have identified *C. difficile* spores in treated water (1), vegetables (1), and the household environment (unpublished data). Exposure to low levels of *C. difficile* might indeed be a common occurrence, with meat being just one of many possible sources. However, recent evidence of increasing rates of CA-CDI, continued identification of *C. difficile* in retail meats, and an overrepresentation of strains found in food animals and food in CA-CDI mean that the potential risks should not be dismissed and that further study of food as a source of infection is warranted.

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## REFERENCES

1. **al Saif, N., and J. S. Brazier.** 1996. The distribution of *Clostridium difficile* in the environment of South Wales. J. Med. Microbiol. **45:**133–137.
2. **Benson, L., X. Song, J. Campos, and N. Singh.** 2007. Changing epidemiology of *Clostridium difficile*-associated disease in children. Infect. Control Hosp. Epidemiol. **28:**1233–1235.
3. **Bidet, P., F. Barbut, V. Lalande, B. Burghoffer, and J. Petit.** 1999. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol. Lett. **175:**261–266.
4. **Hammitt, M., D. Bueschel, M. Keel, R. Glock, P. Cuneo, D. Deyoung, C. Reggiardo, H. Trinh, and J. Songer.** 2008. A possible role for *Clostridium difficile* in the etiology of calf enteritis. Vet. Microbiol. **127:**343–352.
5. **Jhung, M. A., A. D. Thompson, G. E. Killgore, W. E. Zukowski, G. Songer, M. Warny, S. Johnson, D. N. Gerding, L. C. McDonald, and B. M. Limbago.** 2008. Toxinotype V *Clostridium difficile* in humans and food animals. Emerg. Infect. Dis. **14:**1039–1045.
6. **Kato, H., N. Kato, K. Watanabe, N. Iwai, H. Nakamura, T. Yamamoto, K. Suzuki, S. Kim, Y. Chong, and E. Wasito.** 1998. Identification of toxin A-negative, toxin B-positive *Clostridium difficile* by PCR. J. Clin. Microbiol. **36:**2178–2182.
7. **Keel, K., J. Brazier, K. Post, S. Weese, and J. Songer.** 2007. Prevalence of PCR ribotypes among *Clostridium difficile* isolates from pigs, calves, and other species. J. Clin. Microbiol. **45:**1963–1964.
8. **Kuijper, E., J. van Dissel, and M. Wilcox.** 2007. *Clostridium difficile*: changing epidemiology and new treatment options. Curr. Opin. Infect. Dis. **20:**376–383.
9. **Lemee, L., A. Dhalluin, S. Testelin, M. Mattrat, K. Maillard, J. Lemeland, and J. Pons.** 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.
10. **Pirs, T., M. Ocepek, and M. Rupnik.** 2008. Isolation of *Clostridium difficile* from food animals in Slovenia. J. Med. Microbiol. **57:**790–792.
11. **Rodriguez-Palacios, A., R. J. Reid-Smith, H. R. Staempfli, D. Daignault, N. Janecko, B. P. Avery, H. Martin, A. D. Thomspon, L. C. McDonald, B. Limbago, and J. S. Weese.** 2009. Possible seasonality of *Clostridium difficile* in retail meat, Canada. Emerg. Infect. Dis. **15:**802–805.
12. **Rodriguez-Palacios, A., H. Staempfli, T. Duffield, and J. S. Weese.** 2007. *Clostridium difficile* in retail ground meat, Canada. Emerg. Infect. Dis. **13:**485–487.
13. **Rodriguez-Palacios, A., H. Stämpfli, T. Duffield, A. Peregrine, L. Trotz-Williams, L. Arroyo, J. Brazier, and J. S. Weese.** 2006. *Clostridium difficile* PCR ribotypes in calves, Canada. Emerg. Infect. **Dis**. **12:**1730–1736.
14. **Rodriguez-Palacios, A., H. Stämpfli, M. Stalker, T. Duffield, and J. S. Weese.** 2007. Natural and experimental infection of neonatal calves with *Clostridium difficile*. Vet. Microbiol. **124:**166–172.
15. **Rupnik, M.** 2007. Is *Clostridium difficile*-associated infection a potentially zoonotic and foodborne disease? Clin. Microbiol. Infect. **13:**457–459.
16. **Rupnik, M., V. Avesani, M. Janc, C. von Eichel-Streiber, and M. Delmée.** 1998. A novel toxinotyping scheme and correlation of toxinotypes with serogroups of *Clostridium difficile* isolates. J. Clin. Microbiol. **36:**2240–2247.
17. **Songer, J. G., H. T. Trinh, G. E. Killgore, A. D. Thompson, L. C. McDonald, and B. M. Limbago.** 2009. *Clostridium difficile* in retail meat products, USA, 2007. Emerg. Infect. Dis. **15:**819–821.
18. **Spigaglia, P., and P. Mastrantonio.** 2002. Molecular analysis of the pathogenicity locus and polymorphism in the putative negative regulator of toxin production (TcdC) among *Clostridium difficile* clinical isolates. J. Clin. Microbiol. **40:**3470–3475.
19. **Stubbs, S., M. Rupnik, M. Gibert, J. Brazier, B. Duerden, and M. Popoff.** 2000. Production of actin-specific ADP-ribosyltransferase (binary toxin) by strains of *Clostridium difficile*. FEMS Microbiol. Lett. **186:**307–312.
20. **Vengust, M., L. Arroyo, J. S. Weese, and J. Baird.** 2003. Preliminary evidence for dormant clostridial spores in equine skeletal muscle. Equine Vet. J. **35:**514–516.
21. **Weese, J. S., B. Avery, J. Rousseau, and R. Reid-Smith.** 2009. Detection and enumeration of *Clostridium difficile* in retail meat, abstr. P2037. 19th Eur. Conf. Clin. Microbiol. Infect. Dis., Helsinki, Finland.