AEM
Journals.ASM.org

Prevalence of *Clostridium difficile* in Uncooked Ground Meat Products from Pittsburgh, Pennsylvania

Scott R. Curry, Jane W. Marsh, Jessica L. Schlackman, and Lee H. Harrison

Infectious Diseases Epidemiology Research Unit, Division of Infectious Diseases, University of Pittsburgh School of Medicine, and Department of Epidemiology, Graduate School of Public Health, University of Pittsburgh, Pittsburgh, Pennsylvania, USA

The prevalence of *Clostridium difficile* in retail meat samples has varied widely. The food supply may be a source for *C. difficile* infections. A total of 102 ground meat and sausage samples from 3 grocers in Pittsburgh, PA, were cultured for *C. difficile*. Brand A pork sausages were resampled between May 2011 and January 2012. Two out of 102 (2.0%) meat products initially sampled were positive for *C. difficile*; both were pork sausage from brand A from the same processing facility (facility A). On subsequent sampling of brand A products, 10/19 samples from processing facility A and 1/10 samples from 3 other facilities were positive for *C. difficile*. The isolates recovered were inferred ribotype 078, comprising 6 genotypes. The prevalence of *C. difficile* in retail meat may not be as high as previously reported in North America. When contamination occurs, it may be related to events at processing facilities.

The prevalence of *Clostridium difficile* contamination of food products has varied widely, ranging from 0 to 42% (3, 5–10, 14–16, 18–20, 22, 23). The *C. difficile* strains recovered in these studies have included lineages, such as ribotypes 078 and 027, that are commonly encountered in human outbreaks of *C. difficile* infections (CDI), but no epidemiologic connection between human CDI and the food supply has been made (6, 18). Recent evidence suggests either that some *C. difficile* strains are widespread in the food supply or that laboratory contamination led to overestimation of the prevalence of *C. difficile* in some studies (13, 21).

We performed a study of *C. difficile* prevalence in raw retail ground meats in grocery stores in the Pittsburgh, PA, area. Multilocus variable-number tandem-repeat analysis (MLVA), a highly discriminatory genotyping method, was used to evaluate *C. difficile* isolated from food products and to determine the genetic relatedness between *C. difficile* isolates recovered from food and isolates associated with human CDI.

(This work was originally presented in abstract form at the 7th International Conference on the Molecular Biology and Pathogenesis of the *Clostridia* [ClosPath 2011], Ames, IA, 28 October 2011.)

MATERIALS AND METHODS

Sampling scheme. We performed a convenience sampling of 102 raw ground meat products (20 beef, 2 buffalo, 22 chicken, 2 lamb, 41 pork, 10 turkey, and 5 veal) from 3 grocery stores in Pittsburgh, PA. Products in the initial sampling were purchased between 23 February and 28 April 2011. Items purchased included samples of all available deli counter ground meats, all fresh sausages prepared in store, and selected raw, commercially packaged fresh and frozen sausages. Items were kept in their original packaging until the time of sampling. Where available, the USDA establishment number identifying the facility of origin for each product was recorded. Cultures were performed before the sell-by date for each packaged item. Based on the results of the initial sampling, all available brand A pork sausage products were resampled on three separate purchase dates (Table 1).

Microbiological methods. Broth enrichment cultures were performed for all samples as follows: 10 g meat was aseptically transferred to 100 ml cycloserine (500 mg/liter)-cefoxitin (15.5 mg/liter)-mannitol broth with 0.1% taurocholate and 0.5% lysozyme (CCMB-TAL) in 120-ml sterile specimen containers (Starplex Scientific, Etobicoke, Ontario, Canada) and incubated anaerobically at 37°C for 5 days in an anaerobic chamber (Coy Labs, Grass Lake, MI). The CCMB-TAL was not prereduced prior to use. Lids to the specimen containers were loosened to allow for reduction of the liquid medium by exchange with the chamber atmosphere. Visibly fermented samples were subcultured to prereduced Trypticase soy agar with 5% sheep blood (SBA; Becton Dickinson, Franklin Lakes, NJ) and further subcultured to SBA until pure. Colony morphologies on SBA that were consistent with *C. difficile* were confirmed using L-proline aminopeptidase activity (Pro Disk; Remel, Lenexa, KS). A 10-g meat sample spiked with 10 μl of strain CD41 spore stock (~10^5 CFU) served as a positive control for every 8 samples processed. In addition, a CCMB-TAL medium negative control of 100 ml was included for every 8 samples processed.

C. difficile isolates were stored in chopped meat broth (Anaerobe Systems, Morgan Hill, CA). Genomic DNA was extracted after 48 hours of growth of meat broth stocks on brain heart infusion yeast extract agar with 0.1% taurocholate (BHIYT) using an automated magnetic bead extraction platform (NucliSens easyMag; bioMérieux, Durham, NC).

MLVA and *tcdC* genotyping were performed for all isolates (12). The *tcdC* genotype for each isolate was used to infer the ribotype (4). Because two MLVA tandem-repeat loci (CDR4 and CDR5) are absent in inferred ribotype 078 (*tcdC* genotype 20) isolates, the summed tandem-repeat difference (STRD) was calculated using only MLVA loci CDR6, CDR9, CDR48, CDR49, and CDR60. *tcdC* genotypes conform to previously published alleles, with the exception that genotype A/A1 has been renamed genotype 20 to match the *tcdC* genotypes available on the PubMLST website (http://www.pubmlst.org/cdifficile). All isolates with a *tcdC* genotype were presumed to be toxigenic.

The MLVA genotypes recovered were compared to a database of 1,863 isolates collected between 2001 and 2009 from CDI patients and asymptomatic carriers at our institution, including 67 isolates originating from community-acquired, community-onset CDI patients diagnosed between January and June 2011. Food and UPMC clinical isolates were considered highly related if the STRD was ≤2 (11).

Received 13 March 2012 **Accepted** 4 April 2012
Published ahead of print 13 April 2012
Address correspondence to Scott R. Curry, currysr@upmc.edu.

doi:10.1128/AEM.00842-12

TABLE 1 Prevalence of *C. difficile* in brand A pork sausage products, stratified by the facility of origin during initial sampling and during targeted resampling of brand A products

Purchase date(s) (mo-day-yr)	No. positive/no. sampled				Total no. positive/total no. sampled (%)
	Facility A	Facility B	Facility C	Facility D	
February-April 2011	2/2	0/1	0/0	0/0	2/3 (66)
May 14, 2011	4/6	0/4	0/1	0/1	4/12 (33)
August 15, 2011	5/6	1/3	0/0	0/0	6/9 (67)
January 2, 2012	1/7	0/3	0/0	0/0	1/10 (10)
Total	12/21	1/11	0/1	0/1	13/34 (38)

Enumeration of *C. difficile* in positive samples. Quantitative testing of all positive meat samples was performed by direct plating of a 100-μl aliquot from a 1-g meat sample in 9 ml sterile distilled water on CCMB-TAL agar. Because this method would fail to enumerate samples with a contamination density of <100 spores/g, a most-probable-number (MPN) enumeration for 3 positive samples was performed. For the first dilution, 1 g of each sample was cultured in 10 ml CCMB-TAL in each of 5 tubes. For the second and third dilutions, 1,000 and 100 μl, respectively, of a 1-g suspension of meat in 9 ml CCMB-TAL were cultured in 10 ml CCMB-TAL in each of 5 tubes. Each fermenting tube was evaluated for *C. difficile* as above. Estimates of the spore concentration within the original sample were calculated as outlined in appendix 2 of the Food and Drug Administration's *Bacteriological Analytical Manual* (1).

Prevention of cross-contamination in food samples. Samples were kept in their original packaging until laboratory processing and transported in disposable plastic grocery bags.

The following measures were observed to prevent cross-contamination: (i) broth amplification cultures were set up in batches of no more than 25 per day; (ii) samples were set up in a biological safety cabinet in a laboratory separate from the laboratory containing the anaerobic chamber; (iii) all test tube racks, transfer spatulas, and work surfaces were autoclaved on the day of setup; (iv) gloves were changed between each sample; (v) 5,000 ppm sodium hypochlorite was used to disinfect autoclave-intolerant items, including the packages of meat before opening; (vi) the sterile, individually wrapped 120-ml wide-mouthed specimen cups used for broth amplification were kept in sterile overwraps until use; (vii) disposable plastic inoculating loops were used for subcultures; and (viii) meat was kept in a dedicated refrigerator that has never stored *C. difficile* cultures or specimens and is located in a laboratory separate from that used for meat processing and *C. difficile* culture procedures. The selected positive-control strain (CD41) represents a rare MLVA and *tcdC* genotype (2). The CCMB-TAL negative control was set up after processing every eighth sample to monitor for potential laboratory contamination during processing and culture; in addition, the negative-control specimen cup was used as the liquid measuring device for the preceding 8 samples.

RESULTS

Prevalence of *C. difficile* in sampled meat products. Of 102 samples of raw ground meat in the initial sampling (February to April 2011), 2 samples (2.0%; 95% confidence interval [CI], 0.20 to 7.9%) were contaminated with toxigenic *C. difficile* (Table 1). The two positive samples were brand A pork sausages that were purchased on separate dates and cultured in different batches. One of the positive samples was from sausage patties; the other was a 16-ounce sausage chub. Both products originated from the same processing facility (facility A). One other brand A product processed in facility B was negative for *C. difficile* during the initial sampling (Table 1). All CCMB-TAL negative controls were culture negative.

Of 34 brand A products resampled on 14 May 2011, 15 August 2011, and 2 January 2012, 4/12 (33%), 6/9 (67%), and 1/10 (10%) were positive for *C. difficile*, respectively. Of 13 samples positive for *C. difficile* from the entire study, 12 originated from facility A and 1 originated from facility B (Table 1).

Enumeration of *C. difficile* spores in positive samples. Direct plate counts of 13 meat products positive for *C. difficile* were negative for recovery of *C. difficile*. For the three products evaluated by the MPN method, the spore counts were 0.20 (95% CI, 0.01 to 1.0), 0.45 (95% CI, 0.08 to 1.5), and <0.1 (95% CI, <0.18 to 0.68) spores/g.

Genotyping of *C. difficile* isolates. The 13 samples positive for *C. difficile* yielded 14 isolates with 6 distinct *C. difficile* MLVA genotypes (Table 2). One food sample (FD113) yielded two isolates with distinct MLVA genotypes; the second was from an isolate recovered during the MPN procedures. All isolates were inferred ribotype 078, *tcdC* genotype 20 (4). Three MLVA genotypes (A, B, and D) were observed on sampling dates 2, 4, and 5 months apart. The isolate from the product originating in facility B had a unique MLVA genotype distantly related to other genotypes (genotype E) (Table 2).

Relationship between *C. difficile* in food and clinical isolates. Of the MLVA-typed isolates in our database, 24 are *tcdC* genotype 20. One food isolate (FD126) purchased 18 August 2011 was identical by MLVA to the isolate from a CDI case diagnosed 10 years earlier (July 2001). FD27 was highly related (STRD = 2) to 1 other clinical isolate from a patient diagnosed with CDI in 2009. Both patient isolates were categorized as hospital-acquired infections. No patient isolate categorized as community onset or community acquired that was collected between January and June 2011 (n = 67) had a genotype consistent with ribotype 078 (*tcdC* genotype 20).

DISCUSSION

In this study, ground pork products from a single brand were shown to be contaminated with *C. difficile*. Of the 13 contaminated products, 12 originated from a single processing facility. This suggests that *C. difficile* contamination of ground pork products occurred at or before the level of the meat processing facility. The genotypes of all of the isolates were consistent with ribotype 078, a lineage common in food animals (7, 17, 22). Despite MLVA's limited discriminatory power for ribotype 078 (13), 6 unrelated MLVA genotypes were identified among the food isolates. This observation highlights an essential role of MLVA for evaluating the prevalence of *C. difficile* in food. Molecular genotyping methods, such as PCR-ribotyping, do not provide sufficient discrimination to differentiate closely related *C. difficile* isolates. Ribotyping could not discriminate the food isolates in this study and could have led to the erroneous conclusion that identical *C. difficile* strains were present in food products produced in separate processing facilities. MLVA clearly demonstrates the presence of 6 distinct *C. difficile* genotypes in brand A pork products. These data are highly indicative of food-borne *C. difficile* contamination and do not support a laboratory contamination event.

The identification of *C. difficile* with multiple, distinct MLVA genotypes from different ground pork products from a single retail meat processing facility over 5 months suggests probable environmental contamination within that facility. Alternatively,

TABLE 2 MLVA and *tcdC* genotyping results for 14 *C. difficile* isolates from 13 positive meat samples and the positive control[a]

Isolate ID[b]	Purchase date (mo-day-yr)	No. of tandem repeats at each locus					*tcdC* genotype	MLVA genotype
		CDR6	CDR9	CDR48	CDR49	CDR60		
FD27	March 14, 2011	21	12	8	18	2	20	A
FD101	April 28, 2011	35	7	10	17	2	20	B
FD109	May 14, 2011	35	7	10	17	2	20	B
FD110	May 14, 2011	35	14	8	14	2	20	C
FD113	May 14, 2011	35	7	10	17	2	20	B
FD113	May 14, 2011	21	12	8	18	2	20	A
FD117	May 14, 2011	35	7	10	17	2	20	B
FD119	August 15, 2011	28	5	9	27	2	20	D
FD120	August 15, 2011	28	5	9	27	2	20	D
FD121	August 15, 2011	35	9	8	25	2	20	E
FD123	August 15, 2011	28	5	9	27	2	20	D
FD124	August 15, 2011	35	7	10	17	2	20	B
FD126	August 15, 2011	32	12	8	15	2	20	F
FD129	January 2, 2012	28	5	9	27	2	20	D
CD41	NA[c]	18	21	8	16	6	2	G

[a] All isolates are from brand A pork sausage originating from facility A except for the isolate from FD121, which is from brand A pork sausage originating from facility B.
[b] ID, identification.
[c] NA, not applicable.

continuous reintroduction of *C. difficile* from various pig farms or contamination of products handled within the facility by workers who carry *C. difficile* may be occurring.

The level of spore contamination was low (<0.18 to 0.45 spores/g), consistent with a previous study (22). Further research is required to determine whether low levels of spore contamination are able to cause human infections. The low prevalence of ribotype 078, *tcdC* genotype 20 *C. difficile* in hospital- and community-acquired CDI cases from our institution, however, suggests that *C. difficile* in the local food supply may result in only a small proportion of human CDI cases.

Our reported prevalence of *C. difficile* in retail meat was low and in accordance with that found in most previous studies (3, 5, 9, 10, 14–16, 18, 22, 23). Our approach to avoiding laboratory cross-contamination was rigorous and similar in principle to that used in labs performing PCR using dedicated clean rooms.

In summary, we confirmed that *C. difficile* occurs as a low-level contaminant in some uncooked meats and that this contamination may result from events occurring at or before the processing facility. MLVA genotyping of positive samples and other steps to minimize the chances for laboratory cross-contamination should be considered in future studies of the potential contribution of the food supply to *C. difficile* infections in humans. Additional studies to define the contribution of *C. difficile* in food to human CDI are needed.

ACKNOWLEDGMENTS

We thank Mary M. Tulenko for her assistance with the MLVA typing in this work.

Financial support was given by the PA Department of Health (#4100047864, to all authors) and the National Institute of Allergy and Infectious Diseases research career award (K24 AI52788 to L.H.H.).

L.H.H. and J.W.M. have received research grants from ViroPharma and Merck.

REFERENCES

1. **Blodgett R.** 2010. Bacteriological analytical manual appendix 2: most probable number from serial dilutions. US Food and Drug Administration, Washington, DC. http://www.fda.gov/Food/ScienceResearch/LaboratoryMethods/BacteriologicalAnalyticalManualBAM/ucm109656.htm.
2. **Curry SR, et al.** 2007. *tcdC* genotypes associated with severe TcdC truncation in an epidemic clone and other strains of *Clostridium difficile*. J. Clin. Microbiol. **45**:215–221.
3. **de Boer E, Zwartkruis-Nahuis A, Heuvelink AE, Harmanus C, Kuijper EJ.** 2011. Prevalence of *Clostridium difficile* in retailed meat in the Netherlands. Int. J. Food Microbiol. **144**:561–564.
4. **Dingle KE, et al.** 2011. Clinical *Clostridium difficile*: clonality and pathogenicity locus diversity. PLoS One **6**:e19993. doi:10.1371/journal.pone.0019993.
5. **Harvey RB, et al.** 2011. *Clostridium difficile* in poultry and poultry meat. Foodborne Pathog. Dis. **8**:1321–1323.
6. **Harvey RB, et al.** 2011. *Clostridium difficile* in retail meat and processing plants in Texas. J. Vet. Diagn. Invest. **23**:807–811.
7. **Hoffer E, Haechler H, Frei R, Stephan R.** 2010. Low occurrence of *Clostridium difficile* in fecal samples of healthy calves and pigs at slaughter and in minced meat in Switzerland. J. Food Prot. **73**:973–975.
8. **Houser BA, Hattel AL, Jayarao BM.** 2010. Real-time multiplex polymerase chain reaction assay for rapid detection of *Clostridium difficile* toxin-encoding strains. Foodborne Pathog. Dis. **7**:719–726.
9. **Houser BA, et al.** 2012. Prevalence of *Clostridium difficile* toxin genes in the feces of veal calves and incidence of ground veal contamination. Foodborne Pathog. Dis. **9**:32–36.
10. **Jöbstl M, et al.** 2010. *Clostridium difficile* in raw products of animal origin. Int. J. Food Microbiol. **138**:172–175.
11. **Marsh JW, et al.** 2006. Multilocus variable-number tandem-repeat analysis for investigation of *Clostridium difficile* transmission in hospitals. J. Clin. Microbiol. **44**:2558–2566.
12. **Marsh JW, et al.** 2010. Multilocus variable-number tandem-repeat analysis and multilocus sequence typing reveal genetic relationships among *Clostridium difficile* isolates genotyped by restriction endonuclease analysis. J. Clin. Microbiol. **48**:412–418.
13. **Marsh JW, et al.** 2011. Multi-locus variable number tandem repeat analysis for investigation of the genetic association of *Clostridium difficile* isolates from food, food animals and humans. Anaerobe **17**:156–160.
14. **Metcalf D, et al.** 2011. *Clostridium difficile* in seafood and fish. Anaerobe **17**:85–86.
15. **Metcalf D, Reid-Smith RJ, Avery BP, Weese JS.** 2010. Prevalence of *Clostridium difficile* in retail pork. Can. Vet. J. **51**:873–876.
16. **Metcalf DS, Costa MC, Dew WM, Weese JS.** 2010. *Clostridium difficile* in vegetables, Canada. Lett. Appl. Microbiol. **51**:600–602.
17. **Rodriguez-Palacios A, Pickworth C, Loerch S, LeJeune JT.** 2011. Transient fecal shedding and limited animal-to-animal transmission of *Clostridium difficile* by naturally infected finishing feedlot cattle. Appl. Environ. Microbiol. **77**:3391–3397.
18. **Rodriguez-Palacios A, Staempfli HR, Duffield T, Weese JS.** 2007. *Clos-*

tridium difficile in retail ground meat, Canada. Emerg. Infect. Dis. **13**:485–487.
19. **Simango C, Mwakurudza S.** 2008. *Clostridium difficile* in broiler chickens sold at market places in Zimbabwe and their antimicrobial susceptibility. Int. J. Food Microbiol. **124**:268–270.
20. **Von Abercron SM, Karlsson F, Wigh GT, Wierup M, Krovacek K.** 2009. Low occurrence of *Clostridium difficile* in retail ground meat in Sweden. J. Food Prot. **72**:1732–1734.
21. **Weese JS.** 2010. *Clostridium difficile* in food—innocent bystander or serious threat? Clin. Microbiol. Infect. **16**:3–10.
22. **Weese JS, Avery BP, Rousseau J, Reid-Smith RJ.** 2009. Detection and enumeration of *Clostridium difficile* spores in retail beef and pork. Appl. Environ. Microbiol. **75**:5009–5011.
23. **Weese JS, Reid-Smith RJ, Avery BP, Rousseau J.** 2010. Detection and characterization of *Clostridium difficile* in retail chicken. Lett. Appl. Microbiol. **50**:362–365.