MAJOR ARTICLE

Community Environmental Contamination of Toxigenic *Clostridium difficile*

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Background. *Clostridium difficile* infection is often considered to result from recent acquisition of a *C difficile* isolate in a healthcare setting. However, *C difficile* spores can persist for long periods of time, suggesting a potentially large community environmental reservoir. The objectives of this study were to assess community environmental contamination of toxigenic *C difficile* and to assess strain distribution in environmental versus clinical isolates.

Methods. From 2013 to 2015, we collected community environmental swabs from homes and public areas in Houston, Texas to assess *C difficile* contamination. All positive isolates were tested for *C difficile* toxins A and B, ribotyped, and compared with clinical *C difficile* isolates obtained from hospitalized patients in Houston healthcare settings.

Results. A total of 2538 environmental samples were collected over the study period. These included samples obtained from homes (n = 1079), parks (n = 491), chain stores (n = 225), fast food restaurants (n = 123), other commercial stores (n = 172), and hospitals (n = 448). Overall, 418 environmental isolates grew toxigenic *C difficile* (16.5%; *P* < .001) most commonly from parks (24.6%), followed by homes (17.1%), hospitals (16.5%), commercial stores (8.1%), chain stores (7.6%), and fast food restaurants (6.5%). A similar distribution of ribotypes was observed between clinical and environmental isolates with the exception that ribotype 027 was more common in clinical isolates compared with environmental isolates (*P* < .001).

Conclusions. We identified a high prevalence of toxigenic *C difficile* from community environs that were similar ribotypes to clinical isolates. These findings suggest that interventions beyond isolation of symptomatic patients should be targeted for prevention of *C difficile* infection.

Keywords. anaerobic infection; epidemiology; molecular epidemiology; prospective; surveillance study.

Changes in the epidemiology of *Clostridium difficile* infection (CDI) have been dramatic in the United States, Canada, and Europe [1, 2]. *Clostridium difficile* infection has been identified as the most common cause of death due to gastroenteritis in the United States with approximately 14 000 deaths in 2007 [3]. The most recent study from the Centers for Disease Control and Prevention (2011) increased these estimates to 453 000 (95% confidence interval [CI], 397 100–508 500) cases of CDI annually in the United States with an estimated 29 300 deaths (95% CI, 16 500–42 100) [4]. Building on previous studies that identified CDI in non-healthcare settings, 34.2% of CDI cases were considered to be community acquired [5].

Historically, CDI has predominantly been thought to result from recent acquisition of a *C difficile* isolate in a healthcare center. Efforts are primarily directed at prevention of the spread of *C difficile* spores from symptomatic patients to prevent horizontal transmission of spores to other susceptible patients; however, early isolation of colonized patients has been shown to decrease CDI incidence [6]. Case series have suggested a wider source of potential *C difficile* contamination, including water, pets, foods, or farm animals [7, 8]. More recently, a large surveillance study in England demonstrated that a minimum of 45% of *C difficile* strains associated with clinical disease were genetically distinct by whole genome sequencing [9]. The authors concluded that there is a potentially large reservoir of *C difficile* that was most likely environmental in origin. Given these data, and also that the spores of *C difficile* are quite hardy and are able to survive for long periods of time in the environment, we hypothesized that the community setting may contain a large burden of *C difficile* contamination. To test this hypothesis, we conducted a large environmental surveillance study to assess rates of *C difficile* contamination in homes and public areas within Houston, Texas, a large metropolitan city in the southern United States. The objectives of the study were to assess environmental contamination of toxigenic *C difficile* and

to assess strain distribution and cytotoxicity of environmental compared with clinical strains obtained from hospitalized patients with CDI.

METHODS

Environmental Contamination of *Clostridium difficile*

To assess *C difficile* contamination in public areas, common areas of chain merchant stores, fast food restaurants, public parks, and community homes were sampled using presterilized gauze lightly soaked with 0.85% NaCl. To assess *C difficile* contamination in homes, 3 to 5 household items were collected from a convenience sample of community homes located in various parts of Houston, Texas (2013–2015). Houses were identified by placement of advertisments (ads) in a variety of media outlets throughout Houston. Specifically, ads were sent to community and church groups around the city with a request to disseminate the ads during the meetings. The ads included contact information for the investigators who could then explain the purpose of the study. Interested persons were then given instructions on how to perform swabs, or investigators went to the household to perform the sampling. All households were single family or apartment dwellings. No persons in the homes had CDI within the previous 12 months. Surface samples of approximately 1 square foot from common living spaces (kitchen and bathroom) were swabbed with the presterilized cotton gauze. Shoe bottom samples were collected by swabbing the bottoms of a pair of shoes with the same procedure. Cleaning supplies (mops, brooms, dust pans) were also sampled. All samples were collected in a 50-mL size presterilized tube and transported to the laboratory within 12 hours of collection. For other collection areas (merchant stores, fast food restaurants, and public parks), common use areas that were frequently used by the public were targeted (bathrooms, railings, and playground equipment). To compare environmental contamination in hospital versus nonhospital settings, samples were obtained from bathrooms and patient table from hospital rooms with an admitted patient without CDI. For each batch of 10 swabs, a negative control of 1 swab that was not used but placed in with the rest of the swabs was used to assure no cross-contamination of swabs.

Clinical Isolates From Patients With *Clostridium difficile* Infection

Clinical strains of *C difficile* were obtained from a multicenter cohort study in 2 healthcare systems in Houston, Texas (2013–2015) [10]. Seven hospitals served as study sites, including 3 university-affiliated tertiary care centers and 4 community hospitals. In this study, stool specimens from all patients with a stool test positive for *C difficile* as part of routine clinical care were collected. Enzyme-linked immunoassay or a polymerase chain reaction (PCR) detection of the *tcdB* gene in unformed stool was the diagnostic methodology used at all study sites during the study period. Clinical microbiology laboratories would only test unformed stool. Stool samples were further analyzed
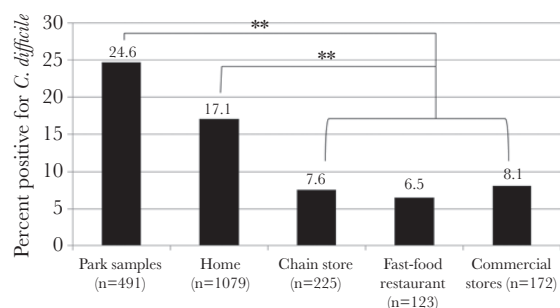
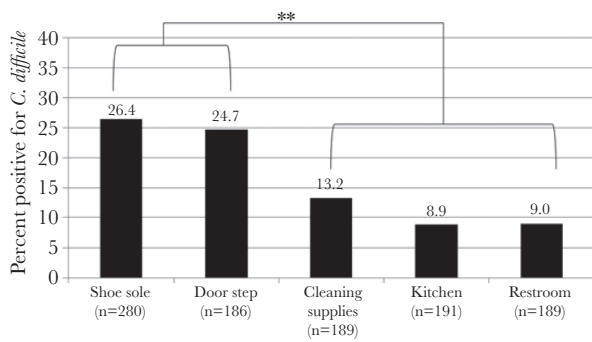**Figure 1.** Community environmental contamination of toxigenic *Clostridium difficile*.

by a centralized research microbiology laboratory for *C difficile* growth, toxin characterization including cytotoxicity, and ribotyping. This study was approved by the Institutional Review Board at the University of Houston and participating hospitals.

Microbiologic Procedures

Stool and environmental samples were enriched in brain heart infusion broth with 0.05% sodium taurocholate (Sigma Chemicals) and incubated anaerobically at 37°C for up to 5 days. One milliliter of broth culture from each sample was centrifuged to concentrate the cells with the resulting pellet suspended in 100 µL normal saline (0.85% NaCl), plated onto cycloserine cefoxitin fructose agar ([CCFA] Anaerobic Systems, Morgan Hill, CA), and incubated anaerobically at 37°C for 40–48 hours (Forma Anaerobic System, Model 1025/1029). Suspected colonies were tested using latex agglutination reagent (Oxoid, Hampshire, England). Each batch of samples was processed with a positive and a negative control. Presence of toxin genes were assessed using multiplex PCR to detect the presence of toxin A (*tcdA*), toxin B (*tcdB*) [11], and the binary toxin (*cdtA* and *cdtB*) genes [12]. Cytotoxicity of all *C difficile* isolates was assessed by the degree of rounding of cells from a Chinese Hamster Ovary cell line (CHO-K1, ATCC CCL-61) as previously described [13].

Clostridium difficile Ribotyping

Fluorescent ribotyping was performed as previously described [14, 15]. In brief, PCR ribotyping primers [16] were synthesized with a fluorescent label (Life Technologies, Inc., Carlsbad, CA) and adjusted to 10 pmol/µL. A 25-µL PCR was performed using AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster city, CA) and the following conditions: 95°C (10 minutes); 35 cycles of 95°C (30 seconds), 55°C (30 seconds), and 72°C (1 minute 30 seconds); final extension of 72°C (10 minutes) (Eppendorf vapo protect thermal cycler, Hamburg, Germany). Amplicons were analyzed using an ABI3730xl DNA Analyzer and MapMaker 1000 ROX DNA sizing standard (BioVentures, Inc., Murfreesboro, TN). Reference strains included PCR ribotypes 027, 001, 053–163, 002, 014–020, 017, and 078, respectively. This technique does not distinguish between ribotypes 053 and 163, ribotypes 014 and 020, and ribotypes 078 and 126;

Figure 2. Home environmental contamination of toxigenic *Clostridium difficile*.

therefore, these are reported as combined ribotypes (ie, 053–163, 014–020, and 078–126).

Clostridium difficile Multilocus Variance Analysis

Multilocus variance analysis (MLVA) typing of *C difficile* isolates ribotype 027 was done using previously published fluorescent MLVA markers [17] using the protocol described previously by Broukhanski et al [18, 19]. In brief, amplicons are diluted in formamide, mixed with LIZ600 deoxyribonucleic acid (DNA) ladder and run on an ABI 3130xl genetic analyzer for fragment analysis. Processing of capillary electrophoresis was done using BioNumerics 7.6 (Applied Maths, Austin, TX) software. Peak files from the genetic analyzer are normalized to the LIZ600 DNA ladder to determine amplicon size. Copy number for each variable number of tandem repeats locus based on amplicon size was determined using previously published guidelines [18].

Analysis

Data were analyzed using SAS, version 9.3 (SAS Institute, Cary, NC). Prevalence of environmental contamination from homes and public space were calculated and compared between sampling

sites using Pearson χ^2. The MLVA dendrograms were created to assess microbial genetics differences between environmental and clinical strains stratified by ribotype. Risk factors for home *C difficile* contamination were assessed using Student *t* test and Pearson χ^2 test as appropriate. A *P* value <.05 was considered significant.

RESULTS

Clostridium difficile Is Highly Prevalent in Environmental Isolates

A total of 2538 environmental samples were collected over the study period. These included samples obtained from homes (n = 1079), parks (n = 491), chain stores (n = 225), fast food restaurants (n = 123), other commercial stores (n = 172), and hospitals (n = 448). Overall, 418 environmental isolates grew toxigenic *C difficile* (16.5%). Prevalence of positive *C difficile* isolates were significantly different based on the location of environmental testing (*P* < .001) (Figure 1). The frequency of *C difficile*-positive isolates was highest in parks (24.6%), followed by homes (17.1%), commercial stores (8.1%), chain stores (7.6%), and fast food restaurants (6.5%). Home environmental contamination with *C difficile* is shown in Figure 2. Prevalence of positive *C difficile* isolates were similar for shoe soles (26.4%), doorsteps (24.7%), and lower for cleaning supplies (13.2%), kitchen areas (8.9%), and restrooms (9.0%). In 448 samples obtained from hospitals, 74 (16.5%) grew toxigenic *C difficile*.

Ribotypes From Environmental *Clostridium difficile* Were Similar to Clinical *C difficile* Isolates

Ribotyping was performed on 344 community environmental toxigenic *C difficile* isolates and compared with 74 hospital environmental isolates and 615 clinical isolates obtained from hospitalized patients with CDI (Figure 3). All toxigenic ribotypes were toxin positive for toxin A and B by PCR. Ribotypes present in at least 10% or more of samples were 014-020 (21.2%),
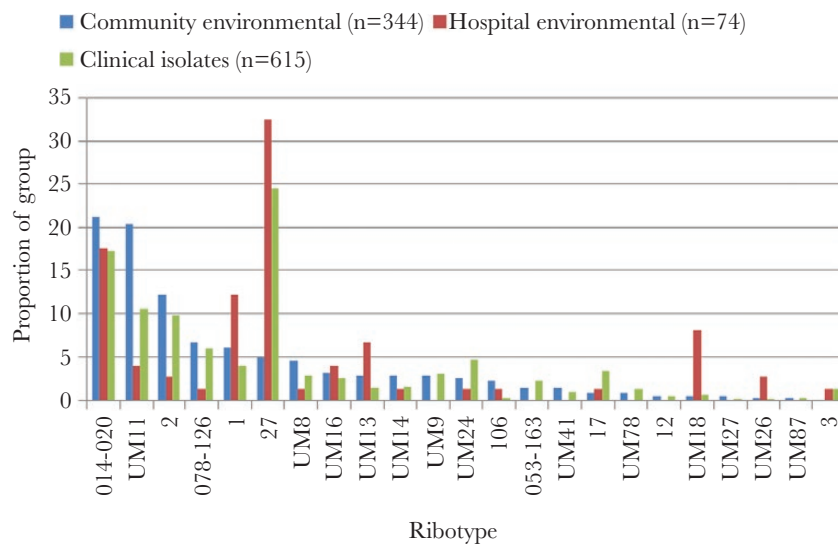
Figure 3. Ribotype distribution of clinical versus environmental *Clostridium difficile* isolates.
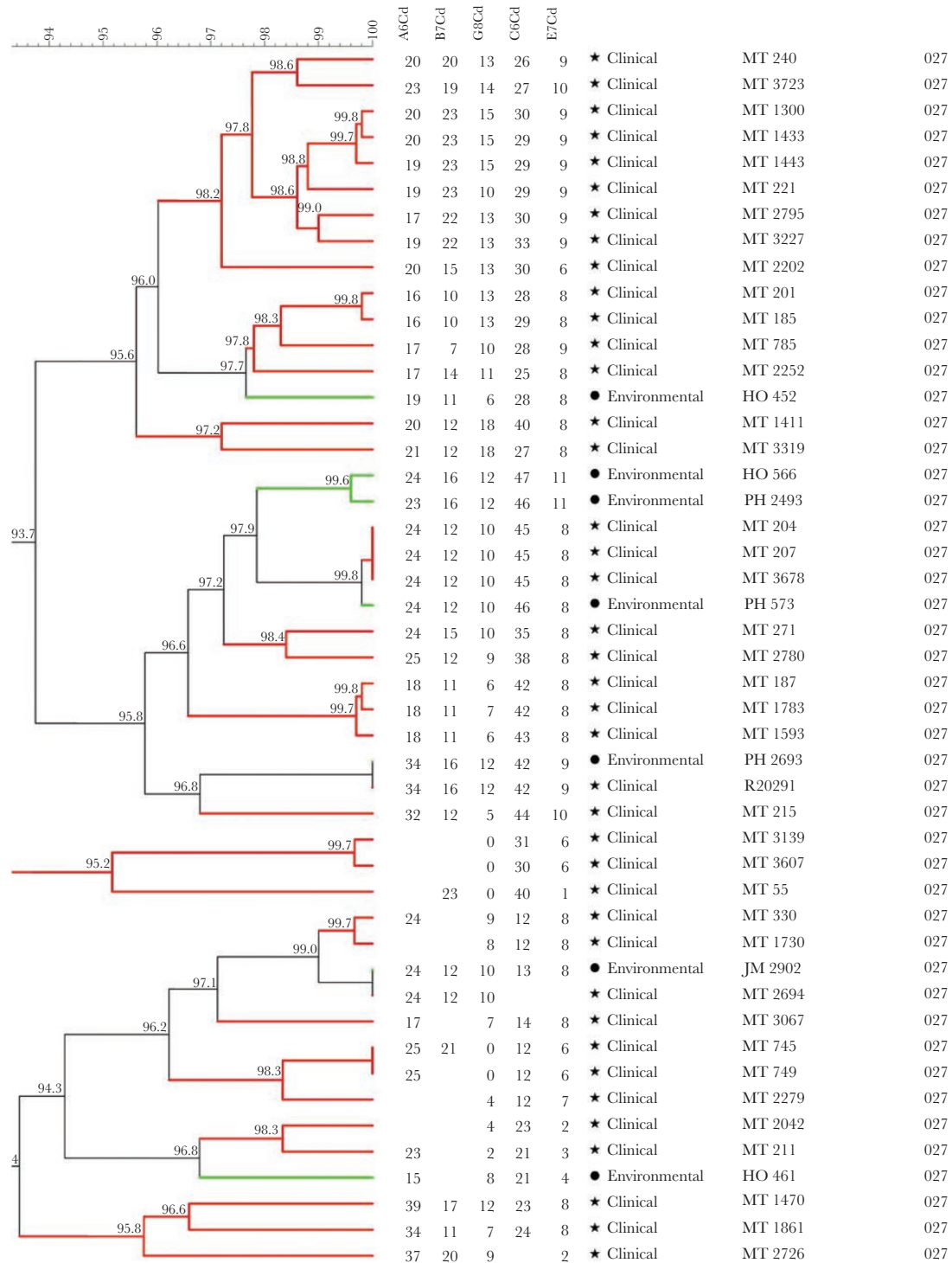
Figure 4. Multilocus variance analysis (MLVA) of environmental and clinical ribotype 027 strains. Environmental strains clustered together with clinical strains.

| A6Cd	B7Cd	G8Cd	C6Cd	E7Cd			
20	20	13	26	9	★ Clinical	MT 240	027
23	19	14	27	10	★ Clinical	MT 3723	027
20	23	15	30	9	★ Clinical	MT 1300	027
20	23	15	29	9	★ Clinical	MT 1433	027
19	23	15	29	9	★ Clinical	MT 1443	027
19	23	10	29	9	★ Clinical	MT 221	027
17	22	13	30	9	★ Clinical	MT 2795	027
19	22	13	33	9	★ Clinical	MT 3227	027
20	15	13	30	6	★ Clinical	MT 2202	027
16	10	13	28	8	★ Clinical	MT 201	027
16	10	13	29	8	★ Clinical	MT 185	027
17	7	10	28	9	★ Clinical	MT 785	027
17	14	11	25	8	★ Clinical	MT 2252	027
19	11	6	28	8	● Environmental	HO 452	027
20	12	18	40	8	★ Clinical	MT 1411	027
21	12	18	27	8	★ Clinical	MT 3319	027
24	16	12	47	11	● Environmental	HO 566	027
23	16	12	46	11	● Environmental	PH 2493	027
24	12	10	45	8	★ Clinical	MT 204	027
24	12	10	45	8	★ Clinical	MT 207	027
24	12	10	45	8	★ Clinical	MT 3678	027
24	12	10	46	8	● Environmental	PH 573	027
24	15	10	35	8	★ Clinical	MT 271	027
25	12	9	38	8	★ Clinical	MT 2780	027
18	11	6	42	8	★ Clinical	MT 187	027
18	11	7	42	8	★ Clinical	MT 1783	027
18	11	6	43	8	★ Clinical	MT 1593	027
34	16	12	42	9	● Environmental	PH 2693	027
34	16	12	42	9	★ Clinical	R20291	027
32	12	5	44	10	★ Clinical	MT 215	027
		0	31	6	★ Clinical	MT 3139	027
		0	30	6	★ Clinical	MT 3607	027
	23	0	40	1	★ Clinical	MT 55	027
24		9	12	8	★ Clinical	MT 330	027
		8	12	8	★ Clinical	MT 1730	027
24	12	10	13	8	● Environmental	JM 2902	027
24	12	10			★ Clinical	MT 2694	027
17		7	14	8	★ Clinical	MT 3067	027
25	21	0	12	6	★ Clinical	MT 745	027
25		0	12	6	★ Clinical	MT 749	027
		4	12	7	★ Clinical	MT 2279	027
		4	23	2	★ Clinical	MT 2042	027
	23	2	21	3	★ Clinical	MT 211	027
	15	8	21	4	● Environmental	HO 461	027
39	17	12	23	8	★ Clinical	MT 1470	027
34	11	7	24	8	★ Clinical	MT 1861	027
37	20	9		2	★ Clinical	MT 2726	027

FP-11 (20.4%), and 002 (12.2%). These results were compared with 615 clinical isolates of *C difficile*. A similar distribution of ribotypes was observed with one exception. Ribotype 014-020 and FP-11 were present in more than 10% of clinical isolates. However, ribotype 027 was much more common in clinical isolates (24.1%) compared with environmental isolates (4.5%; $P < .001$). Similar results were observed with hospital environmental isolates in which ribotype 027 was more common in
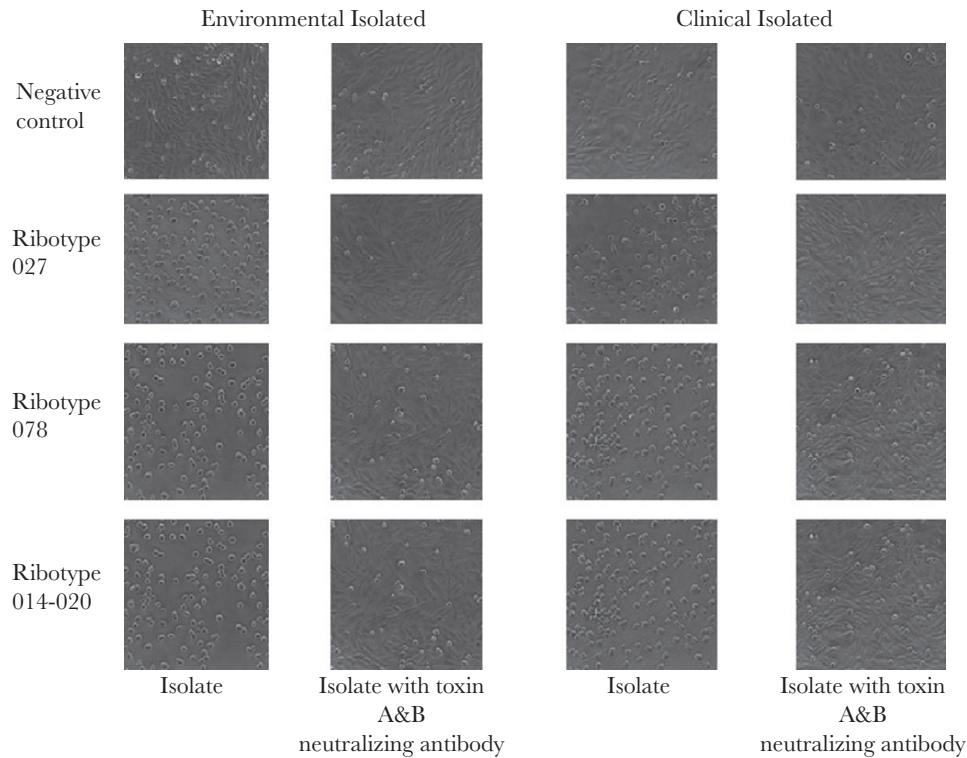
Figure 5. Cytotoxicity of environmental versus clinical isolates by ribotype.

hospital compared with environmental strains. Multilocus variance analysis of ribotype 027 strains showed that there was no genetic distinction between clinical and environmental isolates (Figure 4). All toxigenic isolates identified by PCR (environmental or clinical) were cytotoxic. A representative sample of cytotoxic isolates is shown in Figure 5.

DISCUSSION

In this large surveillance study, between 6.5% and 24.6% of samples obtained from various environmental settings were positive for *C difficile*. Prevalence of *C difficile* was higher in parks and homes and lower in fast food restaurants and merchant stores. Ribotype distribution was similar between environmental and clinical isolates with the exception that the ribotype 027 strain was observed more commonly in hospital clinical and environmental isolates. Strengths of the study include a large sample of environmental samples and comparator clinical isolates obtained in the same geographic location from different hospital types during the same time period. A validated molecular typing method confirmed a similar ribotype distribution between clinical and environmental *C difficile* isolates and other translational research findings including cytoxicity assessment of identified isolates and MLVA analysis.

These results build considerably upon previous findings of *C difficile* in the community environs; specifically, animals and food [20]. Using whole genome sequencing, a recent study demonstrated that 45% of all CDI cases in an endemic environment were attributable to a source unique from any other symptomatic case [9]. A possible community environmental reservoir was posited as the source for these unique strains. A smaller study recently examined household contamination of patients with CDI pre- and postfecal microbiota transplantation [21]. In the posttransplant time period, 38% of household environmental samples were positive for *C difficile*, most commonly the vacuum, toilet, and bathroom sink. In this current study, we demonstrated a high prevalence of *C difficile* isolates in the community comprised of multiple unique ribotypes. The evolutionary dynamics of *C difficile* has shown that it is a genetically diverse species with multiple lineages evolving independently to produce disease [22]. Our results agree with these previous findings because all strains contained toxin genes and were toxigenic using the cell cytotoxicity assay. These findings would suggest that the community environment may be a source of colonization for toxigenic *C difficile*. We have previously shown that half of all patients hospitalized with CDI are diagnosed within 48 hours of admission and up to one third of all cases of *C difficile* infection occur in the community [4, 23]. These findings suggest that specific patient populations at risk for CDI should be targeted for *C difficile* prevention efforts rather than solely focus on containment of patients with active disease. This possibility is re-enforced by previous findings that antimicrobial stewardship efforts can significantly reduce rates

of CDI [24]. This is a paradigm shift in potential prevention efforts for CDI and might shift focus to patient-specific factors to identify a high-risk population. These theories will require further study.

This study has limitations. This surveillance study was conducted in a large, urban area in the southern United States. Results will need to be tested in different geographies. We used a molecular typing method to identify similar ribotypes between clinical and environmental isolates. We used a more discriminatory method (MLVA) for ribotype 027 isolates, but validation with animal models to further discriminate virulence differences was not performed. Our method for *C difficile* detection although sensitive is not quantitative. Whether higher concentrations of spores increases risk of infection is an interesting research question for future work. We did not use traditional random naturalistic home sampling in this study but rather a convenience sample of persons who answered an ad we place at randomly selected community and church group meetings. This study was not intended to identify circulation of *C difficile* isolates between healthcare centers and the community. Likewise, how this spore-forming anaerobe is disseminated throughout the community will require further study.

CONCLUSIONS

In conclusion, we identified a high prevalence of *C difficile* from community environs that were similar ribotypes to isolates that caused clinical disease. These findings suggest that interventions beyond isolation of symptomatic patients should be targeted for prevention of CDI.

References

1. McDonald LC, Killgore GE, Thompson A, et al. An epidemic, toxin gene-variant strain of *Clostridium difficile*. N Engl J Med **2005**; 353:2433–41.
2. Loo VG, Poirier L, Miller MA, et al. A predominantly clonal multi-institutional outbreak of *Clostridium difficile*-associated diarrhea with high morbidity and mortality. N Engl J Med **2005**; 353:2442–9.
3. Hall AJ, Curns AT, McDonald LC, et al. The roles of *Clostridium difficile* and norovirus among gastroenteritis-associated deaths in the United States, 1999–2007. Clin Infect Dis **2012**; 55:216–23.
4. Lessa FC, Mu Y, Bamberg WM, et al. Burden of *Clostridium difficile* infection in the United States. N Engl J Med **2015**; 372:825–34.
5. Chitnis AS, Holzbauer SM, Belflower RM, et al. Epidemiology of community-associated *Clostridium difficile* infection, 2009 through 2011. JAMA Intern Med **2013**; 173:1359–67.
6. Longtin Y, Paquet-Bolduc B, Gilca R, et al. Effect of detecting and isolating *Clostridium difficile* carriers at hospital admission on the incidence of *C difficile* infections: a quasi-experimental controlled study. JAMA Intern Med **2016**; 176:796–804.
7. Lessa FC. Community-associated *Clostridium difficile* infection: how real is it? Anaerobe **2013**; 24:121–3.
8. Hensgens MP, Keessen EC, Squire MM, et al. *Clostridium difficile* infection in the community: a zoonotic disease? Clin Microbiol Infect **2012**; 18:635–45.
9. Eyre DW, Cule ML, Wilson DJ, et al. Diverse sources of *C. difficile* infection identified on whole-genome sequencing. N Engl J Med **2013**; 369:1195–205.
10. Aitken SL, Alam MJ, Khaleduzzuman M, et al. In the endemic setting, *Clostridium difficile* ribotype 027 is virulent but not hypervirulent. Infect Control Hosp Epidemiol **2015**; 36:1318–23.
11. Lemee L, Dhalluin A, Testelin S, et al. Multiplex PCR targeting tpi (triose phosphate isomerase), tcdA (Toxin A), and tcdB (Toxin B) genes for toxigenic culture of *Clostridium difficile*. J Clin Microbiol **2004**; 42:5710–4.
12. Persson S, Torpdahl M, Olsen KE. New multiplex PCR method for the detection of *Clostridium difficile* toxin A (tcdA) and toxin B (tcdB) and the binary toxin (cdtA/cdtB) genes applied to a Danish strain collection. Clin Microbiol Infect **2008**; 14:1057–64.
13. Mattia AR, Doern GV, Clark J, et al. Comparison of four methods in the diagnosis of *Clostridium difficile* disease. Eur J Clin Microbiol Infect Dis **1993**; 12:882–6.
14. Martinson JN, Broadaway S, Lohman E, et al. Evaluation of portability and cost of a fluorescent PCR ribotyping protocol for *Clostridium difficile* epidemiology. J Clin Microbiol **2015**; 53:1192–7.
15. Alam MJ, Anu A, Walk ST, Garey KW. Investigation of potentially pathogenic *Clostridium difficile* contamination in household environs. Anaerobe **2014**; 27:31–3.
16. Bidet P, Barbut F, Lalande V, et al. Development of a new PCR-ribotyping method for *Clostridium difficile* based on ribosomal RNA gene sequencing. FEMS Microbiol Lett **1999**; 175:261–6.
17. van den Berg RJ, Kuijper EJ, van Coppenraet LE, Claas EC. Rapid diagnosis of toxinogenic *Clostridium difficile* in faecal samples with internally controlled real-time PCR. Clin Microbiol Infect **2006**; 12:184–6.
18. Broukhanski G, Low DE, Pillai DR. Modified multiple-locus variable-number tandem-repeat analysis for rapid identification and typing of *Clostridium difficile* during institutional outbreaks. J Clin Microbiol **2011**; 49:1983–6.
19. Broukhanski G, Simor A, Pillai DR. Defining criteria to interpret multilocus variable-number tandem repeat analysis to aid *Clostridium difficile* outbreak investigation. J Med Microbiol **2011**; 60:1095–100.
20. Bauer MP, Kuijper EJ. Potential sources of *Clostridium difficile* in human infection. Infect Dis Clin North Am **2015**; 29:29–35.
21. Shaughnessy MK, Bobr A, Kuskowski MA, et al. Environmental contamination in households of patients with recurrent *Clostridium difficile* infection. Appl Environ Microbiol **2016**; 82:2686–92.
22. He M, Sebaihia M, Lawley TD, et al. Evolutionary dynamics of *Clostridium difficile* over short and long time scales. Proc Natl Acad Sci U S A **2010**; 107:7527–32.
23. Price MF, Dao-Tran T, Garey KW, et al. Epidemiology and incidence of *Clostridium difficile*-associated diarrhoea diagnosed upon admission to a university hospital. J Hosp Infect **2007**; 65:42–6.
24. Sarma JB, Marshall B, Cleeve V, et al. Effects of fluoroquinolone restriction (from 2007 to 2012) on *Clostridium difficile* infections: interrupted time-series analysis. J Hosp Infect **2015**; 91:74–80.