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PCR Detection of *Clostridium difficile* and its Toxigenic Strains in Public Places in Southeast Ohio

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Abstract: *Clostridium difficile* is the most common infectious cause of diarrhea in hospitalized patients with increasing role for the environment as a source of contamination. In this study, we modified a PCR-based approach to investigate the presence of *C. difficile* in environmental soil samples collected from public parks and elementary school playgrounds in Midwestern USA. Out of 246 soil samples collected from 2007 to 2009, 16 (6.5%) were positive for *C. difficile*-specific *tpi* gene amplification. 31.25% of *C. difficile* samples carried both toxin A and B indicating A+B+ strain of *C. difficile* as detected by multiplex PCR targeting *tcdA* and *tcdB* genes. Prevalence of the A+B+ *C. difficile* toxigenic strain ranged between 20-43% during the study period. Despite slight increase in 2009, no significant difference was seen in annual prevalence of the pathogen. The only public sites harboring the toxigenic A+B+ strain of *C. difficile* were the two public parks and no toxigenic strains detected in school playgrounds. Viability of some of the detected spores was confirmed through selective culture. We present a reliable and economic PCR-based approach to screen for *C. difficile* in the public environment that can be adopted to monitor environmental contamination and provide information to assess the risk of occurrence of *C. difficile* in public places.

Key words: Multiplex PCR · Clostridium difficile · Soil · Detection · Toxigenic A+B+ strain · Public Places

INTRODUCTION

Clostridium difficile is a nosocomial bacterium that causes symptoms ranging from diarrhea to life-threatening inflammation of the colon [1,2]. *C. difficile* was recognized as an enteric pathogen in the late 1970s [3] and colonization of *C. difficile* occurs through the oral-fecal route usually after antibiotic therapy that has made the bowel susceptible to infection [4]. Nosocomial *C. difficile* infection, which is associated with diarrhea in about one third of cases, is frequently transmitted among hospitalized patients and the organism is often present on the hands of hospital personnel caring for such patients [5]. Illness from *C. difficile* most commonly affects older adults in hospitals or in long-term care facilities and typically occurs after use of antibiotic medications [6, 7].

These anaerobic, Gram-positive, spore-forming bacilli can produce two types of toxins; toxin A (entero-toxin) and/or toxin B (cytotoxin), leading to mucosal injury and inflammation. Many *C. difficile* variant strains are characterized on the basis of their toxins A and B encoded by *tcdA* and *tcdB* genes respectively [8]. A new strain of *C. difficile* emerged recently in the United States is characterized by increased virulence, resistance to antibiotics or both [9, 10]. *C. difficile* is now considered to be one of the most important causes of health care-associated infections with more infections emerging in the community and in food animals [11]. It is now estimated to infect 3% of healthy adults and 20-30% of hospitalized adults [12].

Few studies have been aimed at detection of *C. difficile* in the environment outside hospitals in an attempt to demonstrate how the general population is exposed to infection. One of the first studied environments was houses of patients with *C. difficile* infection [13]. Additional investigations reported *C. difficile* in several sources including water, soil, feces and gut of livestock animals [14]. Further studies have confirmed presence of *C. difficile* in the environment from Poland [15] and Zimbabwe [16, 17]. Until recently, the bacterium was mainly isolated through culture of its vegetative stage or spores in selective media followed by

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toxin detection with a variety of enzyme immunoassays [18]. However, numerous PCR-based methods have been developed for the detection of *C. difficile* [19]. Lemee and coworkers [20] introduced multiplex PCR diagnostic protocol based on simultaneous amplification of fragments of the triose phosphate isomerase (*tpi*) housekeeping gene and *tcd*A and *tcdB* toxigenic genes of *C. difficile* that allows for detection and toxigenic classification of the pathogen [20].

With increasing number of studies suggesting exposure to the organism in the community [14, 21] and outdoor environment [16], we decided to use this PCR-based approach to investigate the presence of *C. difficile* in environmental soil samples collected from public parks and playgrounds in Midwestern USA to investigate the role of public places as a source for infection.

MATERIALS AND METHODS

Study Area and Sample Collection: Our study area was the Midwest town of Zanesville Ohio, home of the Ohio University campus. Six study sites representing various microenvironments and wide cross section of the local population within the study area were selected among localities of patients of hospital-reported cases of infectious diseases (Fig. 1). These sites included four elementary school playgrounds and two Public Parks (Fig. 1). Except for one school playground site, all sites were within the city limits of Zanesville, Ohio.

Soil samples were collected from specific locations within each of the selected sites in the fall of 2007, spring of 2008 and the fall of 2009. Soil samples (average 20 g) were transferred into sterile 50 ml tubes and transported

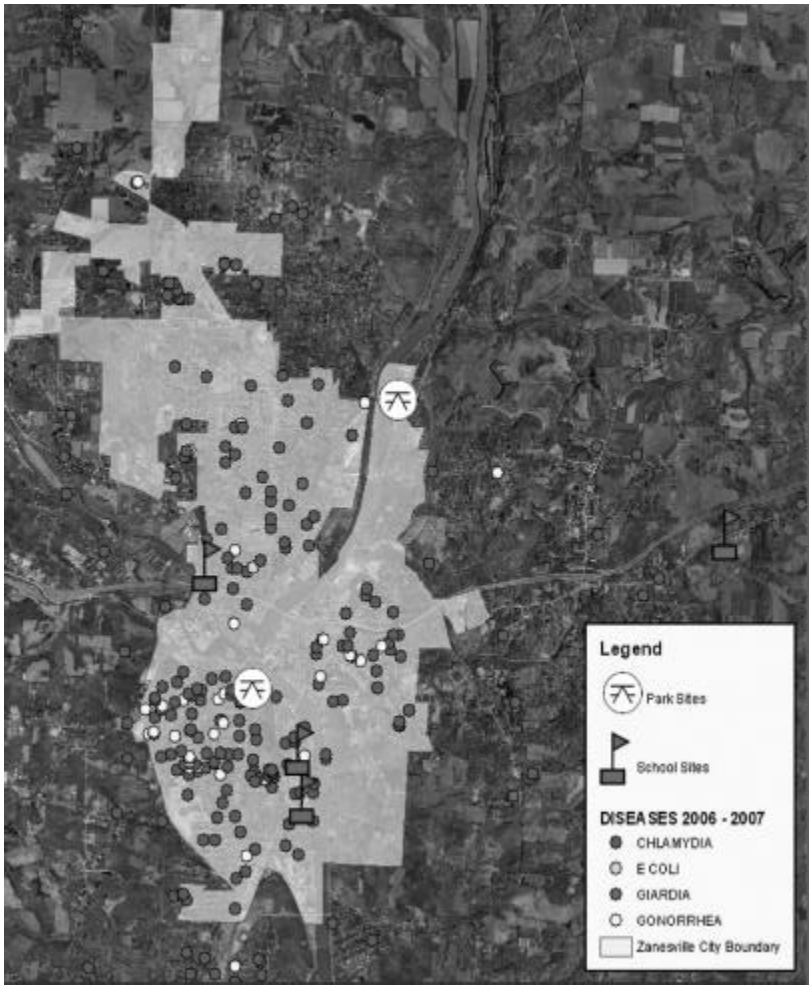

Fig. 1: Map of the study area showing locations of the two public parks and four school playgrounds where samples were collected and distribution of some infectious diseases cases based on local hospital records in 2006/07. Only estimated infectious disease patients locations are shown.

to the laboratory where they were stored at room temperature until further processing. Strict safety guidelines were followed during the collection and transport of the samples.

DNA Extraction and PCR Detection of *C. Difficile* and its Toxins: DNA was extracted from the soil using UltraClean Mega Prep Soil DNA kit followed by PowerClean® DNA Clean-Up Kit or the PowerSoil DNA Isolation kit according to the manufacturer (Mo BIO laboratories, Inc. Carlsbad, CA, USA). The extracted DNA samples were stored at -80°C for PCR use.

Multiplex PCR protocol targeting *C. difficile* and its toxigenic strains [20] was modified by separating the *C. difficile*-specific *tpi* gene amplification step to initially screen for *C. difficile*-specific *tpi* positive samples. Primers, PCR conditions and cycling were the same as those previously reported [20]. PCR products were run on 1.5% agarose to identify 230 bp PCR product specific for *C. difficile*. DNA extracted from *C. difficile* culture was used as a positive control. Isolates that were positive for the *C. difficile*-specific *tpi* assay were then subjected to toxigenic screening by multiplex PCR reaction that allowed for simultaneous detection of toxin A, toxin B and the deleted form of toxin A [20]. Each multiplex PCR reaction contained 2 pairs of the tcdA and tcdB primers that were amplified as reported by Lemee and coworkers [20]. DNA extracted from *C. difficile* culture was used again as a positive control in the multiplex PCR and the products were run on 2% agarose. *C. difficile* strain A+B+ was detected by the presence of two bands of 369 and 160 bp respectively while lack of PCR products indicated the A-B- strain (nontoxigenic) of *C. difficile*.

Viability Testing of C. Difficile: Samples collected in 2009 that yielded positive PCR products were chosen to test viability of the detected *C. difficile* in culture. About five grams of each soil sample were suspended in 10 ml sterile distilled water. A tungsten wire loop was then used to inoculate BBL *C. difficile* selective medium (Becton Dickinson, Sparks, MD, USA) under aseptic conditions. Inoculated plates were immediately incubated in ANAEROJAR AG0025 Anaerobic System (OXOID, Cambridge, UK) supplemented with a sachet that produce anaerobic environment and anaerobic indicator according to the manufacturer. The jar was incubated at 37°C for 60-120 hrs according to culture protocol. *C. difficile* colonies were identified based on shape, color and look under UV light according to BD selective culture medium protocol.

Statistical Analysis: Statistical analysis comparing annual prevalence of *C. difficile* and its toxigenic strains in the study area was performed using Mann Whitney U test [22] of the SPSS version 9 software package (SPSS Inc. Chicago, IL, USA).

RESULTS

From 2007 to 2009, a total 246 soil samples were collected from two public parks and four school playgrounds in Zanesville OH and tested by *C. difficile*-specific *tpi* PCR and multiplex toxigenic PCR. Sixteen samples (6.5%) were positive for *C. difficile*-specific *tpi* gene indicating the presence *of C. difficile* spores in these samples (Table 1). About thirty one percent (31.25%) of *C. difficile* samples carried both toxin A and B indicating A+B+ strain of *C. difficile* (Table 1). The annual breakdown of these isolates and their location within the study sites are shown in Figure 2 and Table 1. While the prevalence of *C. difficile* and its toxigenic A+B+ strain remained the same in 2007 and 2008, an increase in prevalence was seen in 2009 (Fig. 2). However, there was no significant difference in the overall annual prevalence of *C. difficile* and its toxigenic strains ($p > 0.05$) in the study area.

C. difficile was detected in all study sites except one school playground (Fig. 3). However, the toxigenic strain A+B+ was only seen in the two public parks (Fig. 3). Park 2 had both the highest number of *C. difficile* isolates (37.5%), as well as the highest number of the A+B+ toxigenic strain (60%) (Table 1) (Fig. 3). It was also the only site where toxigenic A+B+ *C. difficile* strain has been detected every year (Table 1).

In 2007, 5 out of 77 samples collected in the area were *C. difficile* with one isolate identified as A+B+ strain in Public Park 2 (Table 1) and out of 94 samples screened in 2008, one A+B+ strain identified out of 4 *C. difficile* was found in the same Park. In 2009, 7 out of 96 collected samples contained *C. difficile* with three toxigenic A+B+ isolate, one discovered yet again in Public Park 2 (Table 1). Other two toxigenic A+B+ *C. difficile* isolates were found in two locations in Public Park 1 (Table 1). It is interesting to note that the three toxigenic A+B+ *C. difficile* isolates obtained in three consecutive years from Public Park 2 were collected from locations within 10 feet from each other.

Table 1: Distribution of *Clostridium difficile* and its toxigenic A+B+ strains within the study area from 2007 to 2009 based on multiplex PCR analysis.

Site	Year	*C. difficile*	A+B+ *C. difficile*	Location*	Soil Description*	No. of samples collected
Public Park 1	2007	0	0			14
	2008	0	0			16
	2009	3	2	Perimeter of the basketball court; bottom of the monkey bars; soil below merry go round.	Well graded dry dark brown soil; fragment dry dark brown mulch; coarse moist dark brown peat	8
Public Park 2	2007	2	1	†Near house shoe pit; Picnic bench soil	Fine dry tan soil; fine dry medium brown soil	9
	2008	1	1	†Ground between picnic tables	Medium dry medium brown soil	16
	2009	3	1	†Soil under handrail Soil under picnic table; Soil under a bench	Fine moist black peat; fragment mist anddry dark brown mulch	1 1
Playground 1§	2007	1	0	Along side walk close to chain link fence	Well graded dry gray brown soil	17
	2008	1	0	Soil next to a fence	Well graded dry dark brown soil	17
Playground 2	2007	2	0	Underneath rung of metal rope stairs; soil from pool of rain water under regular side	Fragment saturated dark brown mulch	16
	2008	1	0	In a field	Coarse moist light brown soil	18
	2009	1	0	Next to fence at the edge of the playground	Fine dry tan soil	30
Playground 3	2007	0	0	N/A	N/A	12
	2008	1	0	Soil to entrance of the kids house	Medium dry gray tan soil	14
	2009	0	0	N/A	N/A	15
Playground 4	2007	0	0	N/A	N/A	9
	2008	0	0	N/A	N/A	13
	2009	0	0	N/A	N/A	11
Total	-	16	5	-	-	246

§School playground was demolished late 2008. N/A = not applicable. *Locations and descriptions of soil where A+B+ strain of *C. difficile* was found are listed first, if applicable. †Locations within 10 feet diameter.

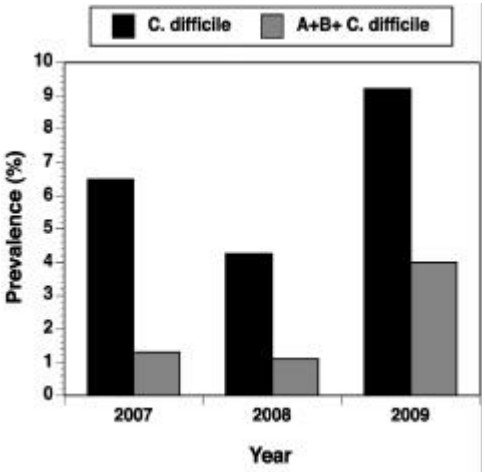

Fig. 2: Prevalence of *Clostridium difficile* and its toxigenic strains in the soil of public places in Zanesville Ohio from 2007 to 2009 based on PCR detection.

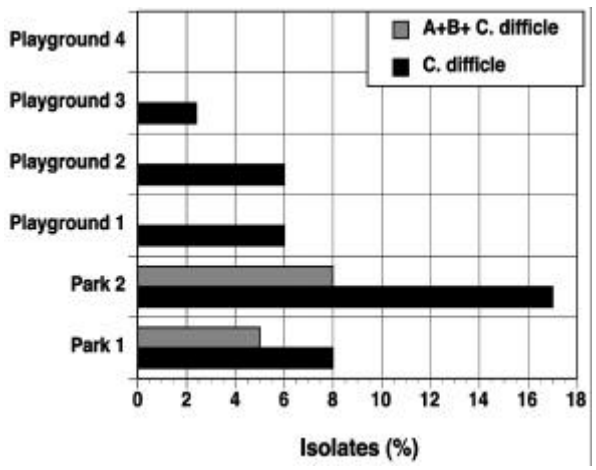

Fig. 3: Prevalence of *Clostridium difficile* and its A+B+ toxigenic strain in four elementary school playgrounds and two Public Parks in Zanesville Ohio from 2007 to 2009.

No correlation was seen between soil type and presence of *C. difficile* (Table 1). Since these *C. difficile* isolates were identified based on PCR, it was essential to investigate the viability of these spores collected from public places. PCR positive samples collected in the fall of 2009 were selected for culture as they represent the

freshest samples. In the fall of 2010, seven soil samples containing *C. difficile* spores based on PCR analysis were cultured in *C. difficile* selective medium as described in Materials and Methods section. Four cultures (57%) grow colonies that were identified as *C. difficile* based on their color, shape and UV fluorescence (data not shown). Initial PCR classification of these cultured *C. difficile* samples were confirmed as three non-toxigenic and one A+B+ toxigenic strain through multiplex PCR performed on DNA extracted from the corresponding cultures (data not shown).

DISCUSSION

Clostridium difficile is an anaerobic, gram-positive, spore-forming, toxin-producing bacillus that is the most common cause of healthcare-associated diarrhea in developed countries [23, 24]. Recent increase in *C. difficile* infections, related hospitalizations and case-fatality rate has been reported [12] and indicate its importance as an emerging infectious agent.

Studies have demonstrated that the environment is an important source of *C. difficile* contamination and that it enhances the potential for the spread of the infection [25] with the spores being capable of persisting on surfaces for prolonged periods [26, 27]. As *C. difficile* is shed in feces, any surface or device that becomes contaminated with feces can serve as a reservoir for its spores [28]. Our finding of *C. difficile* and its toxigenic strains in soil is supported by similar reports from the UK [14], Sweden [29] and Africa [16, 17] and soil from a patient home in the US [13].

Our study demonstrated how human populations can be exposed to *C. difficile* spores on a regular basis in public environment. We also showed steady prevalence of *C. difficile* and its toxigenic strains in the study area over 3 years. In this study, areas with the highest concentration of spores and the only public sites harboring the toxigenic A+B+ strain of *C. difficile* were two public parks. This finding is explained by the fact that *C. difficile* infection is mainly associated with older adults [21, 30] who are disproportionately affected by the disease [31, 32]. This age group constitutes considerable proportion of public parks visitor population compared to the elementary school playgrounds, which were free of toxigenic *C. difficile* in this study. It is also interesting to note that more toxic *C. difficile* was found in Park 2, which has more volume of traffic due to its location within more populated neighborhood.

In Public Park 2, the same toxigenic *C. difficile* spores were detected in locations within 5-10 feet from each other for over 3 years. This might indicate persistence of these spores in soil of public parks for up to 3 years. *C. difficile* spores are highly resistant to drying, heat and chemical and physical agents [12] and can also survive on hospital-inoculated surfaces [13] and in nature and indoors for at least 4 years in inoculated equine feces [29]. However, investigation of the phylogenetic relationship between the toxigenic *C. difficile* isolates [33] detected over the duration of the study period would be needed to verify if these isolates represent a persistent strain in the soil rather than new contaminating spores each year.

Presence of *C. difficile* in the environment in Ohio was evident from a recent study targeting white-tailed deer confinement facilities where 37% of pooled fecal samples were positive [34]. The prevalence of *C. difficile* in public places reported here is similar to that reported from comparable environments [14]. It is also reported that most *C. difficile* isolates recovered from public environment produced no toxins [35]. Only A+B+ toxigenic strain of *C. difficile* was detected in this study. Although strains that produce either toxin A or B exist, most toxigenic strains release both toxins [18, 36, 37]. It would be interesting to follow up the toxigenic strains in the public environment to monitor the effect of environmental conditions and antibiotic exposure on toxin production [38-40] and possible emergence of the hypervirulent strain in the public environment. Our results also suggested that presence of *C. difficile* is not associated with specific soil types and the spores could infest all soil types generally found in Public Parks and playgrounds.

One limitation of our approach is the inability to detect the hypervirulent strain of *C. difficile* known as NAP1/BI/027, which had been implicated in outbreaks associated with increased morbidity and mortality since the early 2000s [41].

The PCR approach used in this study has the advantage of being quick, sensitive and specific [20, 42]. The modification which was made by separating the *C. difficile* specific PCR from the toxigenic multiplex PCR made the assay more economic and practical for screening large number of environmental samples. Our results also showed that the majority of the soil *C. difficile* spores (57%) are accessible for selective media culture confirmation after storage of soil sample at room temperature for over a year. It is expected that more timely confirmation would reveal viability of most if not all collected spores. In this study, the actual production of

toxins A and B by one of the cultured *C. difficile* isolates was not investigated. However, the correlation between detection of *C. difficile tcd*A and *tcd*B genes and production of toxin A and B in culture was confirmed in the original study [20].

The current results showed that *C. difficile* could remain viable in public outdoor environments and support the suggestion of human-to-human transmission within the community. It also emphasized the importance of soil and public environment in acquisition of *C. difficile* in the community. We presented a reliable tool for the screening of *C. difficle* in the environment that can easily be adopted to monitor environmental contamination and provide information to assess the risk of obtaining *C. difficile* in public places.

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